Original Article

Intravitreal Application of Mesenchymal Stem Cell By-Products Does Not Ameliorate Experimental Autoimmune Uveitis

(mesenchymal stem cells (MSCs) / experimental autoimmune uveitis (EAU) / conditioned medium / MSC-derived exosomes / intravitreal administration)

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Abstract. The study aimed to evaluate the therapeutic effects of mesenchymal stem cells and their by-products, including conditioned medium, extract and exosomes, on intraocular inflammation in experimental autoimmune uveitis. Uveitis was induced in the C57BL/6J mouse strain by administration of interphotoreceptor retinoid-binding protein, complete Freund's adjuvant and pertussis toxin. Mesenchymal stem cell-derived products (conditioned medium, extract and exosomes) were prepared and administered intravitreally at varying time points post-induction, primarily on day 14. Retinal inflammatory changes were monitored using fundus imaging, flow cytometry and RT-PCR analysis to evaluate clinical manifestation of inflammation, immune cell infiltration and gene expression of inflammatory markers. *In vivo,* administration of mesenchymal stem cell by-products did not ameliorate experimental autoimmune uveitis. On the contrary, treated eyes exhibited exacerbated inflammation, including increased immune cell infiltration and up-regulated pro-inflammatory gene expression (e.g., *Gfap, Iba1, Il1b* and *Il17*). *In vitro* studies suggested a trend towards anti-inflammatory effects of the conditioned medium, but these findings were not replicated *in vivo*. Control experiments on healthy eyes indicated that intravitreal trauma alone significantly contributed to inflammatory responses, irrespective of the substance injected. Intravitreal application of mesenchymal stem cell by-products did not demonstrate therapeutic benefits in the experimental autoimmune uveitis model and instead promoted inflammation. The results highlight the impact of administration-induced trauma and suggest that alternative delivery methods, such as systemic administration, may be more effective for mesenchymal stem cell-based therapies.

Received September 6, 2025. Accepted January 13, 2026.

This study was supported by the Charles University Institutional Programme GA UK 309621, by the Grant Agency of the Ministry of Health of the Czech Republic, grant number NU23-05-00133, and by the Charles University Institutional Programme SVV 260755.

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Abbreviations: CD – cluster of differentiation, DMEM – Dulbecco's modified Eagle's medium, EAU – experimental autoimmune uveitis, FBS – foetal bovine serum, GFAP – glial fibrillary acidic protein, HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, IBA-1 – ionized calcium-binding adapter molecule 1, IL – interleukin, iNOS – inducible nitric oxide synthase, IRBP – interphotoreceptor retinoid-binding protein, MSC – mesenchymal stem cell, NS – not significant, PBS – phosphate-buffered saline, PTx – pertussis toxin, Rho – rhodopsin, RT-PCR – real-time polymerase chain reaction, SE – standard error, sEVs – small extracellular vesicles, TGF-β – transforming growth factor beta, Th – T-helper.

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Introduction

In developed countries, uveitis represents a significant cause of blindness among individuals of productive age (Tsirouki et al., 2018; Joltikov and Lobo-Chan, 2021), as

 https://doi.org/10.14712/fb2026.0010

the chronic form of intraocular inflammation can lead to irreversible structural alterations, especially in the retina. Effective management of autoimmune uveitis often necessitates combined immunosuppressive therapy, which is associated with significant adverse effects (Rosenbaum et al., 2019), further emphasizing the urgent need for innovative therapeutic approaches to improve the quality of life for affected patients.

Experimental autoimmune uveitis (EAU) serves as a well-established model for studying inflammatory autoimmune diseases affecting the retina and choroid. EAU is typically induced by subcutaneous administration of the retinal antigen interphotoreceptor retinoid-binding protein in complete Freund's adjuvant, accompanied by intraperitoneal pertussis toxin injection (Caspi et al., 1988). The resulting clinical manifestations include vitreous cellular infiltration, choroidal and retinal inflammatory deposits, retinal structural disorganization and perivascular cellular infiltration (Klimova et al., 2016). This model enables *in vivo* monitoring of therapeutic effects using ocular fundus imaging systems and *post-mortem* evaluation of histological sections (Heissigerova et al., 2016).

Stem cell-based therapies, particularly those involving mesenchymal stem cells (MSCs), have garnered significant interest due to their immunomodulatory properties and potential to differentiate into various cell types. MSCs, which can be easily isolated from sources such as bone marrow and adipose tissue, must meet specific criteria defined by the International Society for Cell Therapy. These include the expression of markers such as CD73, CD90 and CD105, lack of haematopoietic markers CD11b, CD14, CD19, CD34, CD45, CD79α and MHC class II molecules, adherence to plastic surfaces in standard culture conditions, and the capacity to differentiate into adipocytes, chondroblasts and osteoblasts (Dominici et al., 2006).

It has been shown that MSCs can differentiate into cells expressing retinal markers (Kicic et al., 2003; Hermankova et al., 2017) and secrete factors aiding retinal regeneration (Kolomeyer and Zarbin, 2014; Park et al., 2017), suppress inflammatory responses in the retina (Rajashekhar et al., 2014; Hermankova et al., 2019) and reduce apoptosis (Mathew et al., 2017). They produce TGF-β and up-regulate immunomodulatory molecules under pro-inflammatory conditions (Hermankova et al., 2019). Systemic MSC delivery has shown potential in treating autoimmune uveitis by modulating immune responses (Li et al., 2013). These properties make MSCs promising for retinal disorder therapies (Holan et al., 2021).

The significant paracrine action of MSCs has drawn growing interest in recent years. Researchers are increasingly exploring the therapeutic potential of the exosomes secreted by MSCs as well as the "conditioned medium", which is the culture medium of MSCs containing their secretory products (Dreixler et al., 2014; Yu et al., 2014, 2016; Bermudez et al., 2016). Mirotsou et al. (2011) were the first to demonstrate, in a rat model of cardiac ischaemia, that the therapeutic effects of MSCs were mediated through paracrine mechanisms. Since this discovery, numerous studies have highlighted the potential of cell-free therapy in regenerative medicine (Ahangar et al., 2020). Within ophthalmology, the application of MSC-derived products has been most extensively studied for corneal diseases (Mansoor et al., 2019). Emerging evidence also indicates the promise of this approach in managing autoimmune intraocular inflammation (Wang et al., 2020).

The EAU model, resembling human autoimmune uveitis, has been investigated only minimally in the context of MSC-derived exosomes and conditioned medium. Shigemoto-Kuroda et al. (2017) used a C57BL/6J mouse model, administering exosomes intravenously one day post-induction as a preventive measure, inhibiting antigen-presenting cells and Th1/Th17 cell activation. Bai et al. (2017) evaluated parabulbar exosome delivery in a rat model, starting nine days post-induction and continuing daily for seven days, reducing lymphocyte infiltration but not T-cell proliferation. Some studies have reported a therapeutic effect of systemic administration of modified MSC-derived exosomes on EAU (Kang et al., 2020; Li et al., 2022; Duan et al., 2024). These results highlight the need for further research on MSC-derived products, focusing on administration protocols and immunomodulatory mechanisms.

Intravitreal drug administration is a widely utilized approach in ophthalmology for the treatment of retinal diseases. This route of administration offers the advantage of delivering therapeutic agents directly to the site of intraocular inflammation, thereby enhancing precision and efficacy compared to systemic intravenous delivery. Furthermore, intravitreal treatments can reduce the frequency of applications and extend the intervals between doses relative to parabulbar administration. In comparison to whole-cell therapies, cell-free approaches using exosomes or conditioned medium are anticipated to provide a superior safety profile, addressing some of the complications associated with the intraocular application of stem cells in clinical settings (Kuriyan et al., 2017).

This study aimed to assess the therapeutic efficacy of MSCs and their by-products in modulating intraocular inflammation, employing the EAU model as a preclinical framework.

Material and Methods

Animals

Female inbred C57BL/6J mice, aged 6–8 weeks, were used in this study. Subjects were sourced from two distinct facilities: the breeding colony at the Center for Experimental Biomodels, First Faculty of Medicine, Charles University, Prague, Czech Republic, for the induction of EAU, and the breeding unit at the Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic, for MSC isolation. All animals were maintained in standard housing conditions with unrestricted access

to food and water and a controlled 12-hour light/dark cycle. Experimental procedures were conducted in accordance with ethical guidelines and were approved by the Commission for Animal Welfare of the First Faculty of Medicine, Charles University, and the Ministry of Education, Youth and Sports of the Czech Republic, in compliance with national animal protection legislation. Ethical approval for this study was granted under protocol number MSMT-17301/2021-5.

Induction of EAU

EAU was induced via subcutaneous injection of 500 μg of interphotoreceptor retinoid-binding protein peptide 1–20 (IRBP; [*Homo sapiens*] H2N-GPTHLFQPSL-VLDMAKVLLD-OH, Vivitide, Gardner, MA) emulsified in complete Freund's adjuvant containing 3.3 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich, St. Louis, MO). Immediately following this procedure, 1.2 μg of pertussis toxin (PTx; List Biological Laboratories, Inc., Campbell, CA) was administered intraperitoneally, as described in previous studies (Klimova et al., 2016).

Isolation of MSCs

Inguinal fat pads from C57BL/6J mice were excised into small fragments and digested for 45 minutes at 37 °C using collagenase I (Sigma-Aldrich) dissolved in Hanks' balanced salt solution (HBSS) supplemented with Ca^{2+} and Mg^{2+}. Following digestion, the resulting cell suspension was centrifuged twice at 250 × *g* for 8 minutes in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10 % foetal bovine serum (FBS; Sigma-Aldrich), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 10 mM HEPES buffer (collectively referred to as complete DMEM).

The single-cell suspension was then seeded into a 75-cm^2 tissue culture flask (Techno Plastic Products, TPP, Trasadingen, Switzerland) containing complete DMEM. After 48 hours of incubation, non-adherent cells were removed through gentle washing, and the adherent cells were further cultivated at 37 °C in a 5 % CO_2 atmosphere. The cultured cells were maintained for one week, and cells from the third passage were harvested and used in subsequent experiments.

Preparation of MSC by-products

Conditioned medium was prepared by culturing MSCs at a concentration of 200,000 cells/ml in serum-free Essential 8 medium (Gibco by Thermo Fisher Scientific, Waltham, MA). After 48 hours of incubation, the medium was collected and centrifuged three times at 12,000 × *g* for 15 minutes to remove cellular debris.

The MSC extract was processed through lyophilization, where cells were frozen at −80 °C in phosphate-buffered saline (PBS) and then centrifuged at 12,000 × *g* for 15 minutes to separate cell membranes. The resulting extract was harvested and utilized in the experiments.

Additionally, standardized lyophilized exosomes derived from human adipose tissue MSCs (Hansa BioMed, Tallinn, Estonia) were prepared at a concentration of 20 μg per 1 μl PBS based on a previously published protocol (Moisseiev et al., 2017).

Cultivation of retinas with MSCs or MSC by-products

MSCs were seeded in 24-well plates at a density of 20,000 cells per well in 1 ml of DMEM. After 24 hours of incubation, the medium was replaced with RPMI 1640 medium (Sigma-Aldrich) supplemented with 10 % FBS, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 10 mM HEPES buffer, referred to as complete RPMI 1640 medium.

Retinas were isolated from mice with EAU on day 21 post-induction, following the removal of the cornea and lens, and cultured either with MSCs or conditioned medium in RPMI 1640 medium. EAU-induced retinas and retinas from healthy mice cultured in the same medium without MSCs or MSC by-products served as controls. To prevent direct contact between MSCs and retinal tissue, the retinas were cultivated on 0.22 μm semipermeable membranes (Nunc, Roskilde, Denmark). After 48 hours of cultivation, retinas were transferred to 500 μl of TRI reagent (Molecular Research Centre, Cincinnati, OH) for RNA extraction and subsequent analysis using RT-PCR.

Intravitreal application of MSC by-products

Conditioned medium, MSC extract, or exosomes were intravitreally administered unilaterally in a volume of 1 μl immediately after preparation. Injections were performed at various time points: day 14; or days 14 and 21; or day 28 post-EAU induction. Control groups included eyes with induced uveitis without intervention, as well as those receiving intravitreal injections of PBS. Additional experiments were conducted on healthy eyes subjected to no intervention, puncture only, or intravitreal injections of PBS, serum-free medium, or conditioned medium.

Intravitreal injections were performed under a microscope using a 5-μl Hamilton syringe (Hamilton, Reno, NV) equipped with a 33 G sharp needle. Before each injection, the syringe and needle were disinfected with a povidone-iodine solution and rinsed with sterile distilled water. The operative field was disinfected with a 2 % chlorhexidine solution for 1 minute, followed by rinsing with sterile distilled water. The injection process lasted 1 minute, and the needle was retained in the wound for an additional minute post-injection to minimize reflux of the injected fluid.

Clinical follow-up after intravitreal administration

The ocular fundus of mice was monitored at regular intervals using an otoscope equipped with a camera and light source, following the method described by Paques et al. (2007). To enhance imaging, a +4-diopter lens was

positioned between the camera and the otoscope. A single image of the posterior central retina from each eye was captured during fundus biomicroscopy. Pupillary dilation was achieved using tropicamide (Unitropic 1 % oph. gtt., Unimed Pharma, Bratislava, Slovakia) and phenylephrine (Neosynephrin-POS 10 % oph. gtt., Ursapharm, Prague, Czech Republic). A carbomer eye gel (Vidisic gel, Bausch and Lomb, Prague, Czech Republic) was applied to the cornea to facilitate otoscope application, as previously described (Seidler Stangova et al., 2019).

Retinal inflammatory changes were evaluated on days 21, 28, or 35 post-induction in the optic disc, retinal vessels and retinal tissue in the posterior fundus (central region) of both eyes (Xu et al., 2008). Scoring was conducted by two independent observers to minimize bias, and a clinical grade for each eye was assigned using a scale of 0 (no detectable changes) to 4 (most severe EAU), based on established criteria (Heissigerova et al., 2016).

For both intravitreal applications and clinical examinations, mice were anesthetized with isoflurane (3 % for induction and 1.5–2 % for maintenance) and kept on a heated bed or pad throughout the procedures. Experiments were concluded on day 28 or 35 post-induction, after which animals were euthanized via cervical dislocation in compliance with the Czech Republic ethical guidelines. The enucleated eyes were further processed for flow cytometry or RT-PCR. Histological analysis was limited to representative samples for morphological verification.

Flow cytometry

Isolated retinas were homogenized and digested at 37 °C in HBSS containing 1 mg/ml collagenase I. After 45 minutes of incubation, the digestion process was halted by adding an excess of RPMI 1640 medium. The resulting cell suspension was filtered and washed via centrifugation at 250 × *g* for 8 minutes.

The retinal single-cell suspension was incubated at 4 °C for 30 minutes with anti-mouse monoclonal antibodies conjugated to allophycocyanin, phycoerythrin, or fluorescein isothiocyanate. Antibodies targeting inflammatory cell markers F4/80, CD3, CD11b, CD45 (all from BioLegend, San Diego, CA) were used (Table 1 in Supplementary materials). To identify dead cells, Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA) was added 10 minutes before analysis.

Data acquisition was performed using an LSRII flow cytometer, and results were analysed with FlowJo 9 software (Tree Star, Ashland, OR). In some cases, retinal samples were transferred into 500 μl of TRI reagent for downstream analysis.

Detection of gene expression by RT-PCR

Total RNA was extracted from MSCs and retinas using TRI reagent, following the manufacturer's instructions. Reverse transcription was performed in the presence of deoxyribonuclease I (Promega, Madison, WI) in DNase I buffer (Promega). Complementary DNA (cDNA) synthesis was carried out using random primers (Promega) and M-MLV reverse transcriptase (Promega) in a reaction mixture with a total volume of 25 μl.

Quantitative RT-PCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a StepOne-Plus Real-Time PCR system (Applied Biosystems). The cycling parameters included an initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 30 seconds and elongation at 72 °C for 30 seconds. Fluorescence data were collected at each cycle following elongation at 80 °C for 5 seconds.

The expression levels of pro-inflammatory factors, including GFAP, IBA-1, IL-1α, IL-1β, IL-6, IL-17, iNOS, and a marker for photoreceptors rhodopsin (Rho) were quantified (Table 2 in Supplementary materials). Data analysis was performed using StepOne Software version 2.3 (Applied Biosystems). Relative gene expression levels were normalized to the expression of the reference gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*).

Data analysis

Data were analysed using *R: A language and environment for statistical computing* (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/). The results are expressed as the arithmetic mean ± SE (standard error). Mann-Whitney non-parametric tests were used to evaluate differences between the groups. The P value of < 0.05 was considered statistically significant.

Results

Culture of retinas with EAU in the presence of MSCs or MSC-conditioned medium

In vitro cultivation of EAU retinas (obtained on day 21 post-EAU induction) in the presence of MSCs showed a trend towards reduced relative gene expression of *Iba1* (a marker for activated macrophage/microglia) and *Il1b* (a pro-inflammatory cytokine) compared to culture in medium without MSCs, although these changes did not reach statistical significance (Fig. 1). Similarly, in the presence of MSC-conditioned medium, a non-significant trend towards reduction in the relative gene expression of *Iba1*, *Il1b* and *Il6* (a marker of inflammatory response) was observed compared to culture in the same medium without MSC-secreted products (Fig. 1).

The trend towards reduction in the relative gene expression of markers *Iba1* and *Il6* appeared more pronounced in EAU retinas cultivated in the presence of MSC-conditioned medium compared to those co-cultured directly with MSCs. These findings suggest that MSC-conditioned medium might possess a greater potential to modulate the inflammatory response than MSCs.

Interestingly, the gene expression of *iNOS*, a typical macrophage marker, showed no significant changes under *in vitro* conditions (Fig. 1).

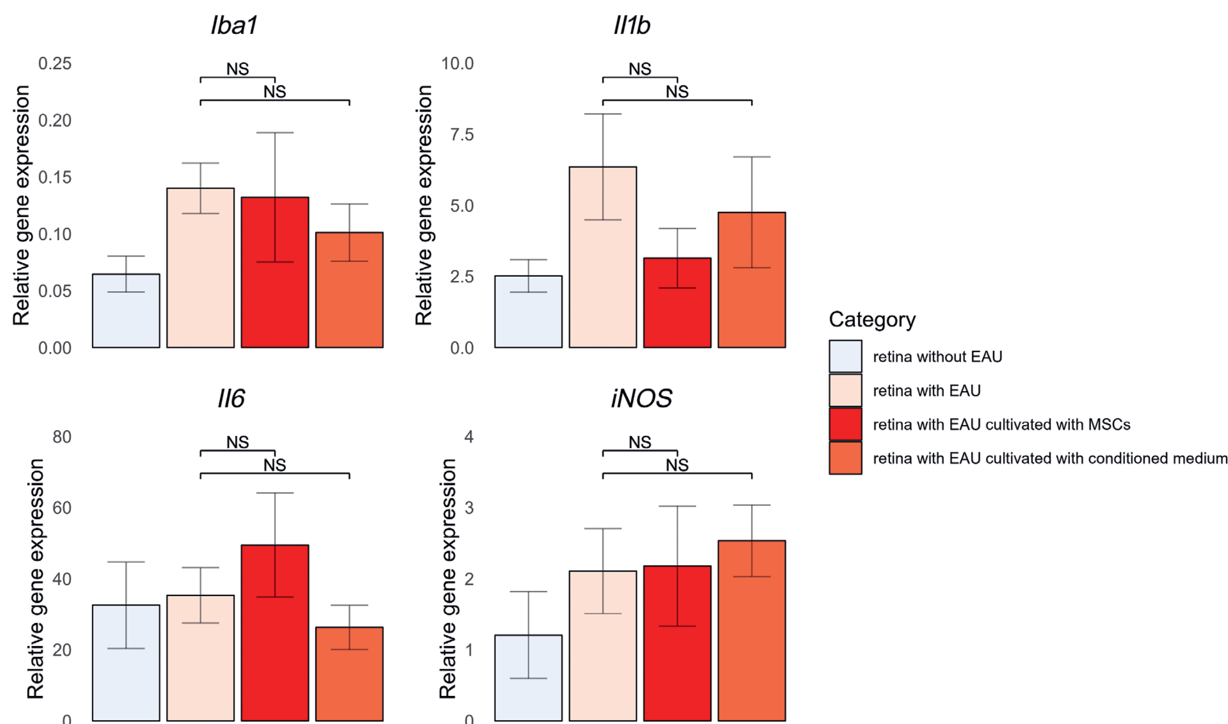

Fig. 1. Relative gene expression of selected factors expressed by EAU-induced retinas cultivated *in vitro* with MSCs or MSC-conditioned medium. Retinas with EAU were obtained on day 21 post-EAU induction and cultivated for 48 hours. EAU-induced retinas and retinas from healthy mice cultured in the same medium without MSCs or MSC-secreted products served as controls. Retinas were isolated from EAU mice exhibiting moderate clinical signs (maximum clinical grade of 2). Consequently, the elevation of inflammatory markers in the positive control group was mild compared to naïve controls. NS (not significant)

Effect of conditioned medium on the course of EAU

Based on the results of *in vitro* experiments, we proceeded to evaluate the therapeutic potential of MSC-secreted products in subsequent *in vivo* experiments. Conditioned medium, obtained by culturing MSCs in serum-free medium for 48 hours, was administered into the right eye on day 14 post-EAU induction.

Ocular fundus examination on days 21 and 28 post-induction revealed no improvement in EAU clinical grading in the treated eyes compared to untreated controls. In some cases, the intraocular inflammation was even more pronounced in the treated eyes (Fig. 2A, B). The average score at both time intervals was higher in the group of injected eyes compared to eyes with no manipulation. The score was increasing over time in both groups.

Flow cytometry analysis of CD45 (an immune cell marker), CD3 (a T-lymphocyte marker) and F4/80 (a macrophage marker)-positive cell populations, performed on day 28 post-induction, showed no positive effect on immune cell infiltration in the treated eyes compared to untreated controls (Fig. 3A, 3B). Additionally, RT-PCR analysis of *Iba1* revealed higher expression levels in the eyes with intervention compared to controls, suggesting a potential exacerbation of inflammatory responses (Fig. 3C).

Effect of extract from homogenized MSCs on the course of EAU

Based on the adverse effects of the conditioned medium on intraocular inflammation, we proceeded to test an alternative approach – MSC extract, prepared by centrifugation of MSCs and removal of cellular membranes. The extract was administered to the right eye on day 14 or days 14 and 21 post-EAU induction.

Clinical evaluation on days 21 and 28 post-induction revealed an increased inflammatory immune response in the treated eyes compared to control eyes (Fig. 4A, B). The score was increasing over time in all groups. The average score at both time intervals was higher in the groups of injected eyes compared to eyes with no manipulation. Worsening of intraocular inflammation was statistically significant on day 21 in the group after two applications and on day 28 in both groups.

Similarly, flow cytometry analysis on day 28 post-induction demonstrated no reduction in immune cell infiltration in the eyes treated with the extract compared to untreated controls. Given the detection of F4/80 and the expression of *Iba1* in previous experiments, we further investigated whether the infiltrating cells contributing to the increased inflammation were predominantly macrophage-like (CD11b, CD45 high) or microglial-like (CD11b, CD45 low) populations. The results indicated a

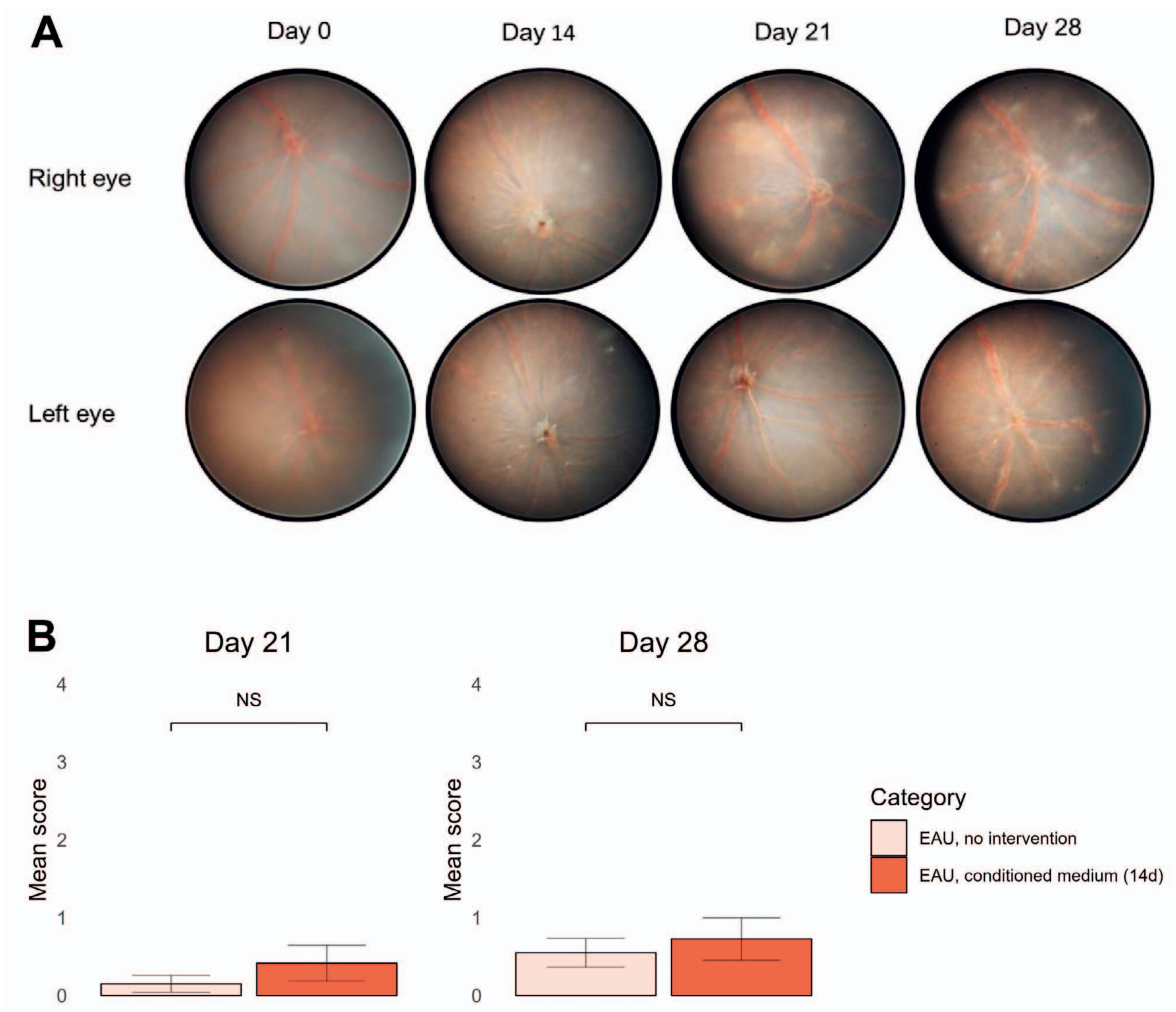

Fig. 2. **(A)** As an example, bilateral intraocular findings of a mouse with EAU before and after conditioned medium administration. Conditioned medium was administered into the right eye on day 14 post-EAU induction. A photo on day 14 was taken before the procedure. Typical signs of uveitis, including chorioretinal lesions and vessel sheathing, were observed in both eyes on day 28 post-induction. However, the onset of these symptoms occurred earlier in the treated eye compared to the untreated eye. **(B)** Clinical grading on days 21 and 28 post-EAU induction. Conditioned medium was administered unilaterally into the mouse vitreous on day 14 post-induction. On days 21 and 28, all eyes were assigned with clinical score (0–4); results were compared to contralateral eyes with no intervention.

higher percentage of CD11b, CD45 low cells in the treated eyes, suggesting a greater prevalence of microglial-like cells. The change in the number of the T-cell ($CD3^+$) population was also measured (Fig. 5A, B).

Effect of MSC-derived exosomes on the course of EAU

The results of the previous *in vivo* experiments indicate that neither the conditioned medium nor the MSC extract improved EAU outcomes; on the contrary, both interventions were associated with a deterioration in the condition.

Based on these findings, we shifted our focus to the administration of exosomes. Standardized lyophilized exosomes from human MSCs from adipose tissue were applied intravitreally into the right eye at a concentration of 20 µg per 1 µl PBS on day 14 or 28 post-EAU induction. The application of exosomes did not reduce the development of inflammation, and in most cases, the eye with applied exosomes developed more pronounced vasculitis than the eye without treatment (Fig. 6A, B). The average score at both time intervals was higher in the group treated with exosomes compared to eyes with no manipulation. The score was increasing over time in both groups.

RT-PCR analysis was limited to specific genes that could contribute to the development of inflammation – *Gfap* (a marker of activated astrocytes), *Iba1*, *Il17* (a pro-inflammatory cytokine, produced by Th17 lymphocytes)

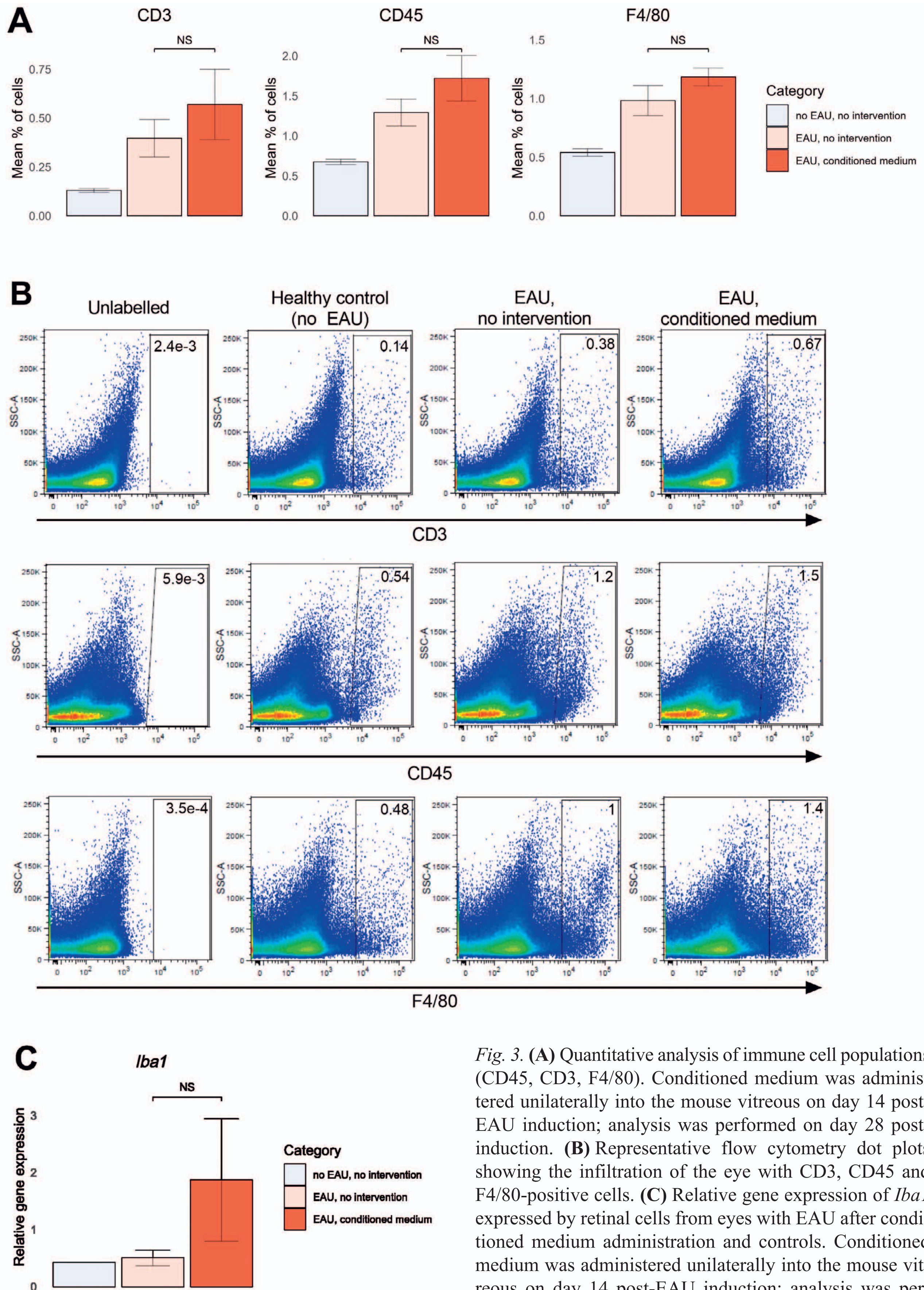

Fig. 3. **(A)** Quantitative analysis of immune cell populations (CD45, CD3, F4/80). Conditioned medium was administered unilaterally into the mouse vitreous on day 14 post-EAU induction; analysis was performed on day 28 post-induction. **(B)** Representative flow cytometry dot plots showing the infiltration of the eye with CD3, CD45 and F4/80-positive cells. **(C)** Relative gene expression of *Iba1* expressed by retinal cells from eyes with EAU after conditioned medium administration and controls. Conditioned medium was administered unilaterally into the mouse vitreous on day 14 post-EAU induction; analysis was performed on day 28 post-induction.

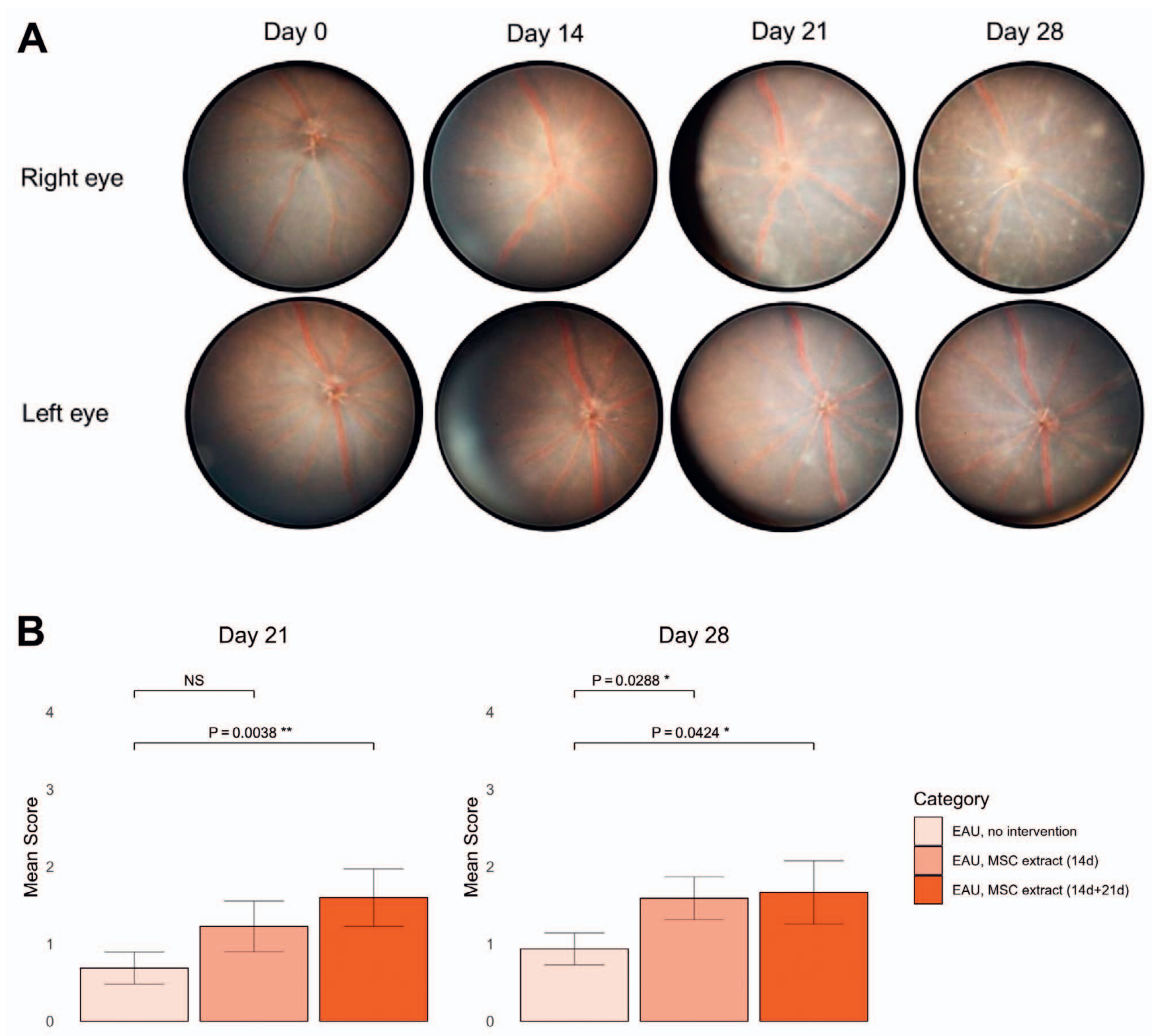

Fig. 4. **(A)** Representative images of bilateral intraocular findings of a mouse with EAU before and after MSC extract administration. The MSC extract was administered into the right eye on day 14 post-EAU induction. A photo on day 14 was taken before the procedure. Typical signs of uveitis such as chorioretinal lesions and vessel sheathing were more pronounced in the treated eyes. **(B)** Clinical grading on days 21 and 28 post-EAU induction. The MSC extract was administered unilaterally into the mouse vitreous on day 14 post-induction (group EAU, extract (14d)) or twice on days 14 and 21 (group EAU, extract (14d+21d)). On days 21 and 28, all eyes were assigned with clinical score (0–4). Results were compared to contralateral eyes (group EAU, no intervention).

and *Il1b*. These genes were selected based on previous results, where microglia and macrophage populations were the most prominent according to flow cytometry (with microglia being more prominently represented according to the CD11b, CD45high/low distribution) together with a T-lymphocyte population. Analysis was performed on day 28 post-induction when exosomes were administered on day 14 post-induction (Fig. 7A), or day 35 when exosomes were administered on day 28 post-induction (Fig. 7B). At both time intervals, the results indicate exacerbation of intraocular inflammation in the eyes with intravitreally administered exosomes compared to controls.

Control experiments on healthy eyes

Considering the previously found adverse effect of MSC by-products on intraocular inflammation, the toxicity of the medium and the technical design of intraocular application on the course of intraocular inflammation were investigated.

Conditioned medium was administered into the vitreous of the right eye of healthy mice; control groups were eyes with injection of no substance (puncture) and contralateral eyes with no intervention. RT-PCR analysis was performed on day 14 post-intravitreal injection. Compared to eyes with no intervention, there was a decrease in pro-

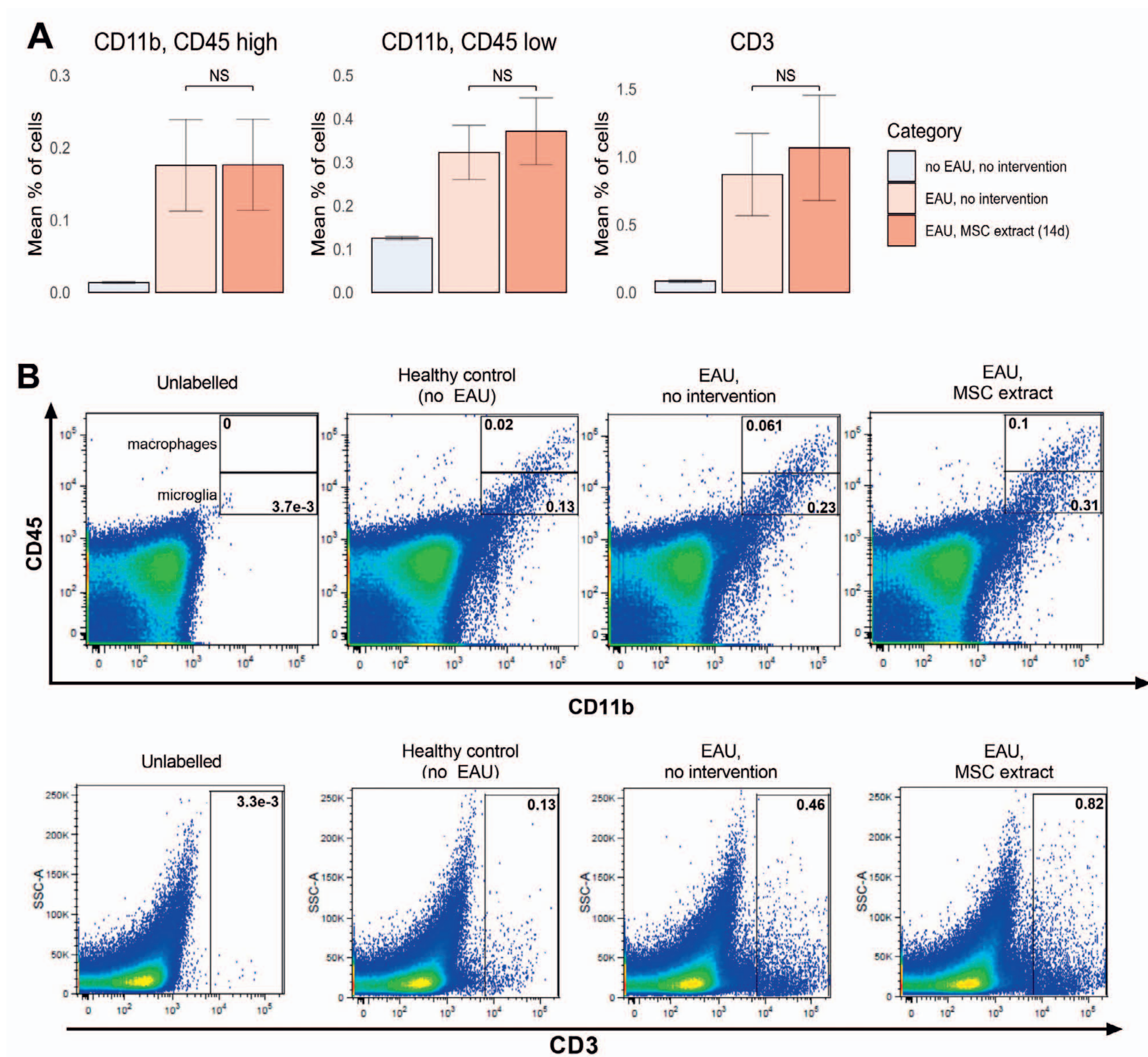

Fig. 5. **(A)** Flow cytometry analysis of eyes with EAU after MSC extract administration and controls. The MSC extract was administered unilaterally into the mouse vitreous on day 14 post-EAU induction; analysis was performed on day 28 post-induction. **(B)** Representative flow cytometry dot plots.

inflammatory factors in the group with a conditioned medium; upsurge in pro-inflammatory factors was noted in the group with puncture only (Fig. 8A).

During another experiment, the gene expression of *Rho* (a marker for photoreceptors) and *Iba1* was monitored as markers of retinal damage after the invasive procedure (Fig. 8B). The application of serum-free medium was used as a control for the conditioned medium and MSC extract. The administration of PBS was used as a control for experiments using exosomes.

The results showed that immediately after the invasive procedure, there was an increase in *Iba1* expression with a peak on day 7 after the procedure and a decrease again on day 21. The results between the groups with injection only and the groups with actual intravitreal application of PBS or serum-free medium do not appear to be significant. *Rho* expression did not appear to be affected by the invasive procedure. The results suggest that the medium in which MSC by-products are suspended probably does not have a significant effect on retinal atrophy and accentuation of the development of intraocular inflammation. The rise and subsequent fall in *Iba1* is probably related to invasive entry into the eye and is furthermore not potentiated by the presence of PBS or serum-free medium in the vitreous.

Discussion

This study investigated the therapeutic efficacy of MSCs and their by-products in the context of EAU, utilizing a well-established preclinical model. Despite the promising immunomodulatory properties attributed to

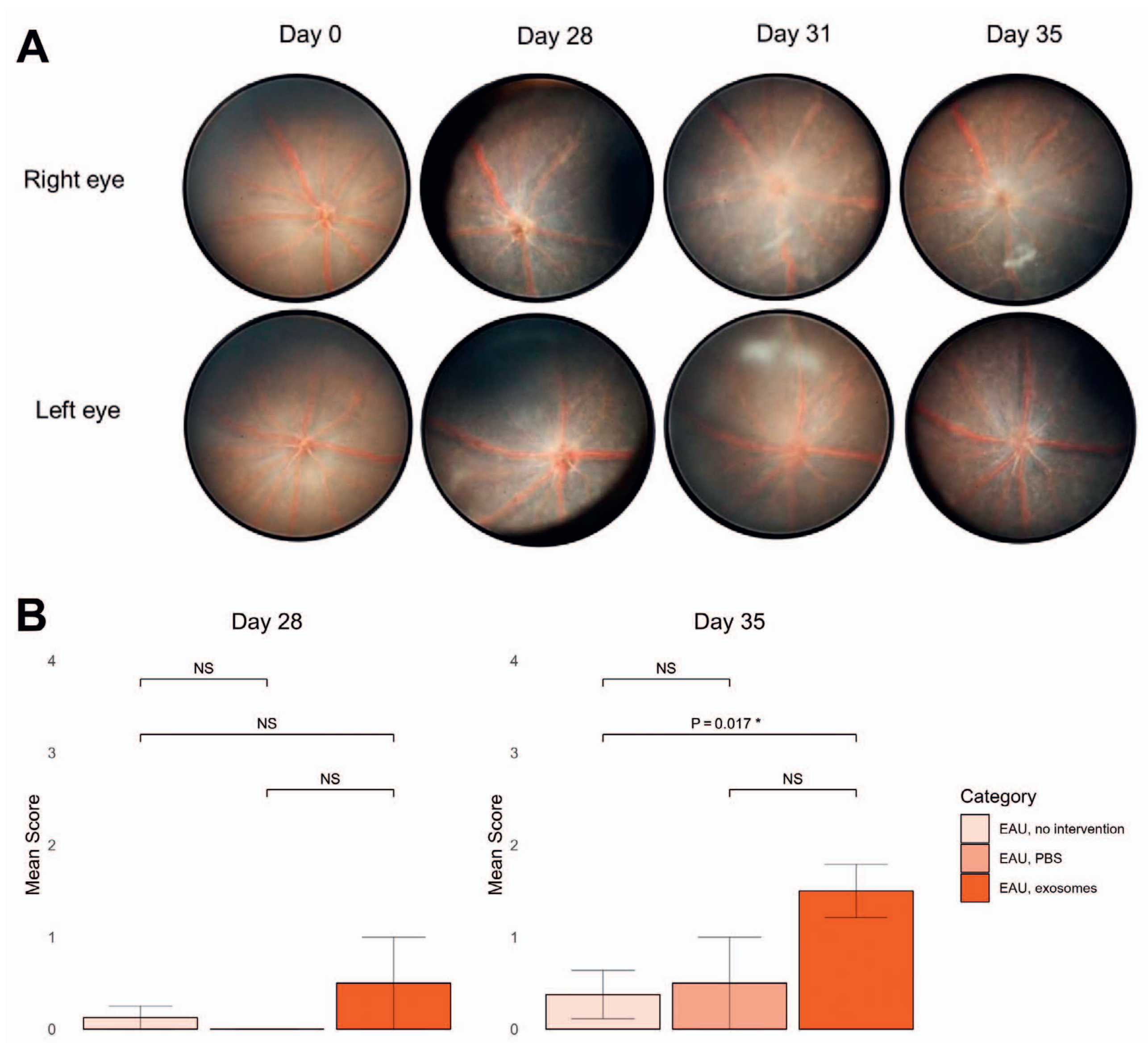

Fig. 6. **(A)** Intraocular findings of eyes with EAU before and after MSC exosome administration. MSC exosomes were administered into the right eye on day 28 after EAU induction. A photo on day 28 was taken before the procedure. **(B)** Clinical grading on days 28 and 35 post-EAU induction. Exosomes were administered unilaterally into the mouse vitreous on day 28 post-induction. On days 28 (before the procedure) and 35, all eyes were assigned with clinical score (0–4). Results were compared to contralateral eyes with no intervention and eyes with PBS application.

MSCs and their secretory products, the results demonstrated that intravitreal application of conditioned medium, MSC extract and MSC-derived small extracellular vesicles (sEVs, exosomes) did not yield the anticipated amelioration of intraocular inflammation associated with EAU. In fact, clinical assessments revealed an exacerbation of inflammatory responses following treatment, as evidenced by increased clinical grading and heightened expression of pro-inflammatory markers.

In contrast, other studies have reported promising results with systemic administration. For instance, MSC-derived sEVs with over-expressed CD73 or IL-10 have demonstrated significant immunosuppressive effects in EAU models when administered via tail vein injection, resulting in reduced inflammation and improved clinical outcomes (Li et al., 2022; Duan et al., 2024). The systemic administration of MSCs has been shown to decrease the development of severe EAU by modulating immune responses, such as suppressing Th17 cells and enhancing Treg cells (Tasso et al., 2012; Qin et al., 2018).

While systemic administration of MSC by-products shows potential, the intravitreal approach may introduce additional variables. In our study, although the *in vitro* experiment suggested a trend towards anti-inflammatory effects, across all *in vivo* experiments, an increased infiltration of the retina by inflammatory cells and an increase in the gene expression of pro-inflammatory factors such as GFAP, IBA-1, IL-1β and IL-17 were observed. Control

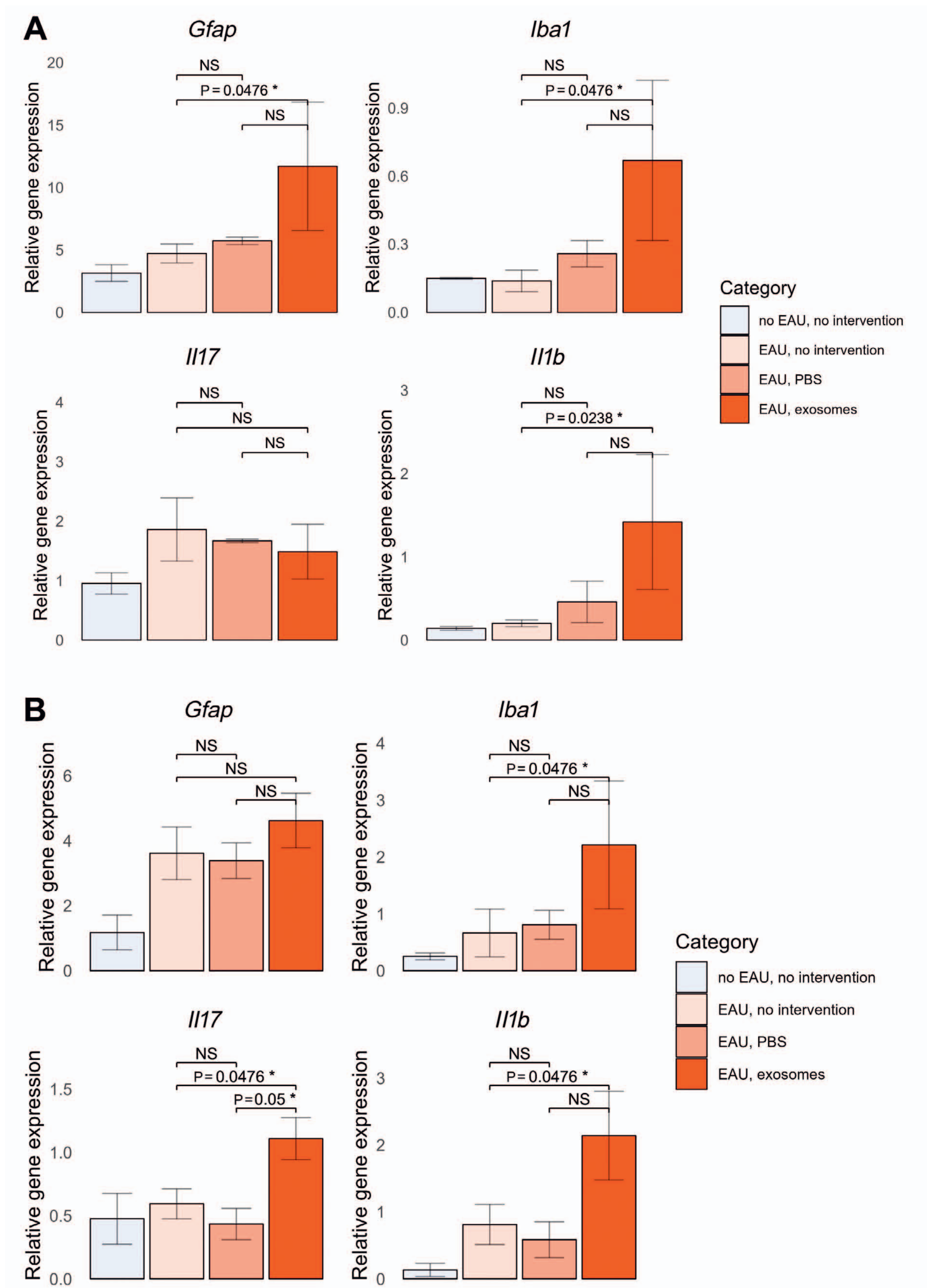

Fig. 7. Relative gene expression of selected factors expressed by retinal cells from eyes with EAU after exosome administration and controls. **(A)** Exosomes were administered unilaterally into the mouse vitreous on day 14 post-EAU induction; samples were collected and processed on day 28 post-induction. **(B)** Exosomes were administered unilaterally into the mouse vitreous on day 28 post-EAU induction; samples were collected and processed on day 35 post-induction.

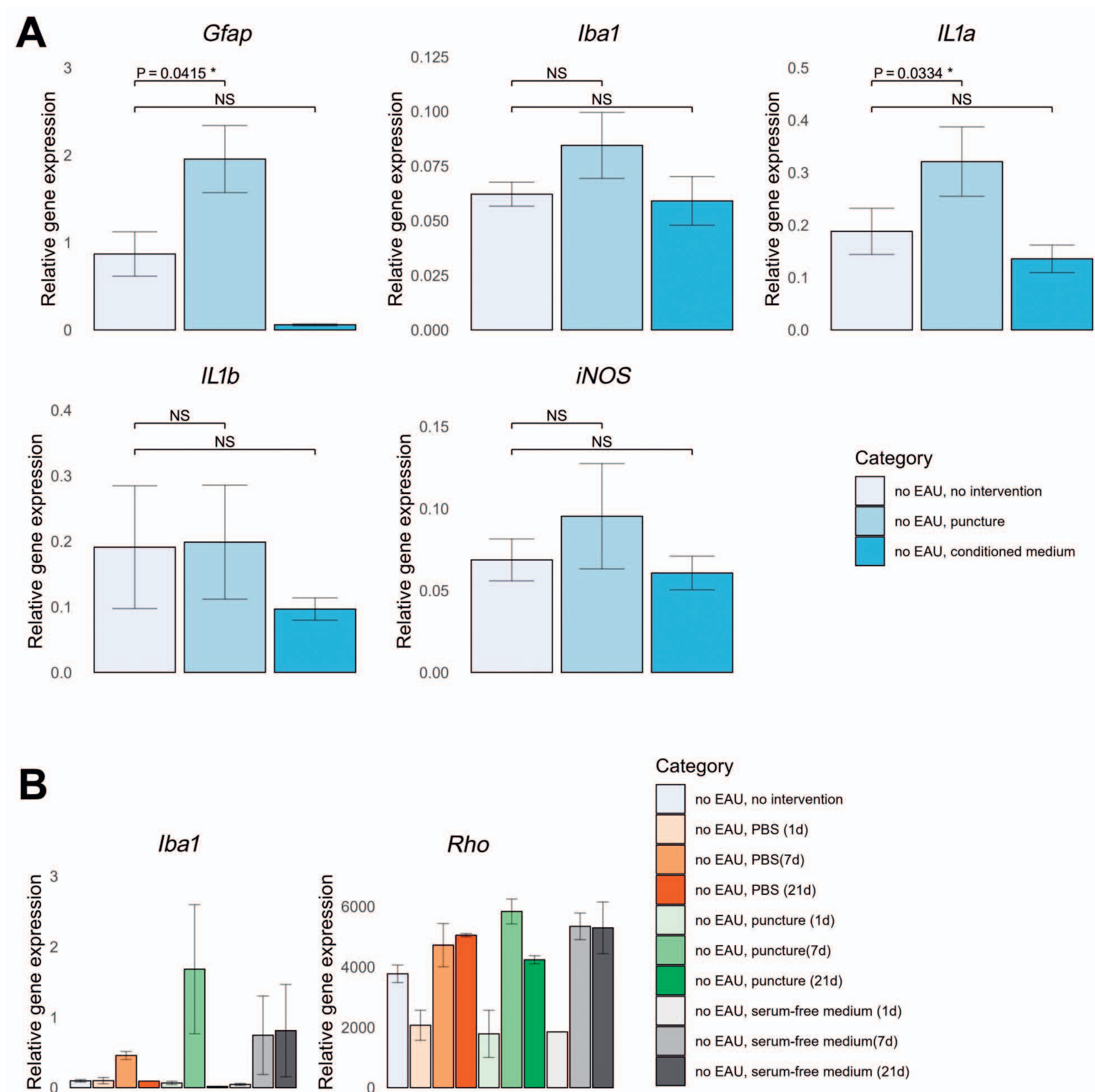

Fig. 8. Toxicity control of auxiliary media and technical performance. **(A)** Relative gene expression of selected factors expressed by retinal cells from healthy eyes 14 days after conditioned medium administration or puncture only or no intervention. **(B)** Relative gene expression of *Rho* and *Iba1* were monitored as markers of retinal damage after the invasive procedure 1 day, 7 days and 21 days after intervention.

experiments on healthy eyes showed an increase in pro-inflammatory factors following intravitreal injection of no substance. The results show the significance of the influence of trauma caused by the intravitreal application itself. Comparing our results from the therapeutic intervention (Fig. 7) with the toxicity controls (Fig. 8), we observed that the elevation of pro-inflammatory markers (*Iba1*, *Il1b*) following a simple intravitreal puncture in healthy eyes reached levels comparable to those seen in the eyes with EAU. This indicates that the invasive procedure itself generates a significant inflammatory signal that may rival the intensity of mild-to-moderate autoimmune uveitis, thereby complicating the assessment of therapeutic efficacy.

Beyond mechanical trauma, specific variables related to the exosome treatment likely contributed to the adverse outcomes. First, the protein concentration (20 μg in 1 μl), although selected based on protocols previously shown to be neuroprotective in retinal ischaemia (Moisseiev et al., 2017), may have induced a sterile inflammatory response in the hypersensitive context of EAU. Second, the use of human-derived exosomes in a murine model represents a xenogeneic application. Although MSC-derived sEVs are generally regarded as hypoimmunogenic and

capable of cross-species therapeutic effects (Li et al., 2022), the introduction of human proteins into an eye with active autoimmune uveitis might have triggered immune recognition. This xenogeneic factor, combined with the high local concentration and mechanical trauma, likely potentiated the inflammatory milieu.

The timing of therapy also plays a crucial role in its efficacy, as demonstrated not only in the case of MSCs but also in other areas such as the administration of probiotics. Studies have shown that early therapeutic interventions, such as those administered close to the induction of a pathological process, yield significantly better outcomes compared to interventions applied during the established phase of inflammation. For instance, probiotics administered near the induction of inflammation effectively modulated the inflammatory response, whereas administration 14 days post-induction was considerably less effective (Dusek et al., 2020). Shigemoto-Kuroda et al. (2017) administered MSC-derived exosomes intravenously one day post-EAU induction. This approach primarily served as a preventive intervention rather than a therapeutic strategy. Bai et al. (2017) utilized a rat model of EAU to evaluate the parabulbar administration of MSC-derived exosomes at the first clinical signs of uveitis. Initial doses were typically administered on day 9 post-induction, followed by daily parabulbar applications for seven days. These findings suggest that late application of MSCs or their derivatives may be less capable of disrupting established inflammatory processes.

Some studies have reported improved therapeutic effects with repeated therapy administration (Zhao et al., 2016; Bai et al., 2017). While intravitreal delivery is expected to achieve higher local concentrations of the therapeutic substance compared to intravenous and parabulbar application, it still raises the question of whether repeated applications could enhance efficacy and, if so, what the optimal timing intervals should be. In our experiment, where the MSC extract was administered twice, on days 14 and 21 post-induction, improved outcomes were not observed. This suggests that either the timing of administration, the frequency, or the specific characteristics of the MSC extract may require optimization.

In conclusion, the results of our observations did not confirm the hypothesis of a reduction in the intensity of intraocular inflammation. No therapeutic effect on EAU was observed following the intravitreal application of MSC by-products. Intraocular application of MSC by-products did not induce the expected immunosuppressive reaction; on the contrary, the development of an inflammatory reaction was promoted. The trauma induced by intravitreal application itself significantly influenced the outcome. According to the available literature, other routes of administration appear more promising than intraocular application. Future investigations should focus on optimizing delivery techniques and further elucidating the underlying mechanisms of MSC action to advance the field of regenerative medicine in ophthalmology. Continued exploration of these strategies will be essential in identifying the most effective protocols for patient treatment, aiming to save sight and improve the quality of life for those affected by sight-threatening retinal diseases.

Ethical guidelines statement

All procedures related to use of animals were conducted in compliance with all relevant EU laws and ethical guidelines and approved by the institutional ethical committee.

Conflict of interest

The authors have no conflict of interest to declare.

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