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Original Research

Subconjunctival injection of mesenchymal stromal cells protects the cornea in an experimental model of GVHD

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ABSTRACT

Purpose: To evaluate the therapeutic effect of subconjunctival injection of human mesenchymal stromal cells (hMSCs) in the cornea of mice with graft versus host disease (GVHD).

Methods: GVHD was induced in mice after hematopoietic stem cell transplantation (HSCT) between MHC-mismatched mouse strains. Subconjunctival injection of hMSCs was applied at day 10 post-HSCT. Infiltration of $CD3^+$ cells in the cornea and epithelial alterations were analyzed by immunofluorescence. Tear was assessed using the PRT test and TearLab Osmolarity System. qPCR was used to evaluate changes in cytokines, Pax6 and Sprr1b expression. To evaluate the effect of irradiation, we analyzed the expression of these genes in TBI mice.

Results: Immune cell invasion occurs in mice with GVHD, as shown by the presence of $CD3^+$ cells in the cornea. Interestingly, eyes treated with hMSC did not present $CD3^+$ cells. Tear osmolarity was increased in GVHD eyes, but not in treated eyes. TNFa expression was highly increased in all corneas except in Control and treated eyes. Pax6 in corneal epithelium showed a similar pattern in GVHD and Control mice, and its gene expression was enhanced in GVHD corneas. In contrast, Pax6 was reduced in GVHD + MSC corneas. We also found an increase in SPRR1B staining in GVHD eyes that was lower in GVHD + MSC mice, demonstrating that corneal keratinization is less frequent after treatment with hMSC.

Conclusions: The treatment with hMSCs by subconjunctival injection is effective in reducing corneal inflammation and squamous metaplasia in ocular GVHD (oGVHD). Local treatment with hMSCs is a promising strategy for oGVHD.

1. Introduction

Graft versus host disease (GVHD) is a common complication of hematopoietic stem cell transplantation (HSCT), an established and potentially curative treatment for hematological disorders [1]. Ocular involvement is present in 60–90% of patients [2]. This ocular GVHD (oGVHD) affects the ocular surface and lacrimal glands, as well as the Meibomian glands, leading to tear film instability. The consequences of this tear film instability include disruption of the epithelial barrier [3,4], pain, irritation and impaired vision [5]. All this makes ocular problems one of the main causes of loss of quality of life in patients with GVHD [6,7].

Although murine models of GVHD have been largely used, it has been only recently when researchers have paid more attention to oGVHD. Our group has previously reported ocular surface damage in a GVHD mouse model, characterized by the presence of lymphocytic infiltration [8], as well as tear disturbances [9]. Moreover, lacrimal gland involvement in a similar GVHD murine model has been demonstrated [10]. In another study, authors showed the recruitment of donor T lymphocytes in recipient mice after MHC-matched HSCT [11]. Therefore, models of mouse GVHD seem a good tool to broaden the knowledge of GVHD and to develop new treatments.

As the current treatment of oGVHD is palliative, usually based in tear substitutes, it is necessary to explore new treatments to improve the prognostic of this debilitating disease. Suppression of the immune response has shown some promising results [2,12], but

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immunosuppressive drugs like tacrolimus are not recommended for long-term use [1] and some patients just do not respond to treatment [13]. The use of mesenchymal stromal cells (MSC) as a cell-based therapy is a promising strategy for the treatment of a number of conditions [14–17]. Given their ability to limit tissue destruction and enhance repair in various diseases, some researchers have used them for reparative treatments in animal models, including for corneal damage repair [18–24]. In the last years, their immune-modulatory effects have received special attention and they are being used in a number of immune-mediated diseases with promising results, including GVHD [25–28]. However, as most of these studies have not passed phase-III clinical trials, it is necessary to extend our knowledge on the biological effects of MSCs and to identify their mechanism of action in disease states.

In the present study we have used a well-established murine model of oGVHD [9] combined with in vitro experiments with immortalized human corneal-limbal epithelial (HCLE) cells. We have extended our knowledge on the corneal consequences of oGVHD in mice, such as squamous metaplasia, T cell invasion and cytokine production. In addition, we have tested the curative potential of hMSCs, showing an improvement in the parameters analyzed.

2. Material and methods

2.1. Animals

All maintenance and manipulation protocols were conducted in compliance with the guidelines of the Council of European Communities (Directive 2010/63/EU) and Spanish legislation (R.D. 53/2013) for the use and care of laboratory animals in the Animal Experimentation Service of the University of Salamanca. Also, all projects using animals were approved by the Animal Ethical Committee of the University of Salamanca before initiating any procedures (Ethical Committee permission no. JLR/bb) and firmly adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

BALB/c and C57BL/6J mice of 8–10 weeks of age were purchased from Charles River Laboratory (France) and were housed with ad libitum access to food and water in specific pathogen-free conditions. All the experiments were performed under the same controlled and standardized conditions of room temperature, airflow and humidity.

2.2. Hematopoietic stem cell transplantation

Transplantation was performed as previously described [8,9,29], using 4×10^6 splenocytes. C57BL/6 ($H2^b$) male mice were used as donors and BALB/c ($H2^d$) female mice were used as recipients. Briefly: recipient BALB/c mice received total body irradiation (850 cGy divided in two fractions) from a Cs source (GammaCell 1000, Nordion International, Ottawa, ON, Canada). Irradiation was followed by the intravenous infusion of 5×10^6 C57BL/6 allogeneic donor bone marrow (BM) cells with or without splenocytes (4×10^6 cells intravenously) as a source of allogeneic T cells.

2.3. GVHD assessment

The presence of signs of systemic GVHD was assessed as described by Cooke et al. [30]. Hence, five parameters were considered: weight loss, posture (hunching), activity, fur texture and skin integrity. Each parameter received a score from 0 to 2 attending to severity, giving a total range from 0 to 10.

2.4. Subconjunctival injection of hMSCs and experimental groups

Human MSCs were isolated from healthy human donors as previously described [29]. Briefly, BM was taken by iliac crest aspiration under local anesthesia and mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare Bio-Sciences, Uppsala, Sweden) and cultured in standard culture medium (DMEM; Gibco) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. After the third passage, hMSCs were assessed following the minimal criteria recommended by the International Society for Cellular Therapy (ISCT).

At day 10 post-transplantation, some mice received an injection with hMSCs. For the injection, mice were anesthetized by isoflurane inhalation (Abott Laboratories). We instilled one drop of topical anesthetic in the eye before injection. We administered 2×10^5 hMSCs in 20 µl of PBS injection on the right eye with a 27G Terumo Myjector (Terumo Europe). After injection, we instilled one drop of ciprofloxacin (3 mg/ml; Alcon).

The following experimental groups were used: *Control* group (non-manipulated mice), *TBI* group (non-transplanted irradiated mice), *BM* group (mice irradiated and transplanted only with BM), *GVHD* group (mice irradiated and transplanted with BM cells and splenic cells), *GVHD + MSC* group (hMSCs-treated eyes (right eye) from mice irradiated and transplanted with BM cells and splenic cells) and *GVHD + MSC LE* (untreated contralateral eyes (left eyes) from animals treated with hMSCs after irradiation and transplantation with BM cells and splenic cells).

2.5. Immunofluorescent staining

At day 28 post-transplant (18 days post-hMSCs injection), animals were euthanized and the eyes were enucleated. After fixation overnight at 4 °C in 4% paraformaldehyde corneas were separated, cryoprotected in 30% sucrose and frozen in OCT®. Sections of 12 µm were obtained in a cryostat (Microm HM560, Thermo Scientific). Four to five eyes per group were used.

Sections were permeabilized with 0.02% Triton X-100 in PBS (PBS-TX) and blocked with 5% donkey serum in the same medium for 1 h at room temperature (RT). Then, sections were incubated overnight with rabbit anti-CD3 (1:200; ab5690, Abcam), anti-Pax6 (1:500; PRB-278P, Covance) or anti-SPRR1B (1:200; SAB1301567, Sigma-Aldrich) antibodies, diluted in the blocking mixture, at RT. After washing in PBS-TX, sections were incubated with donkey anti-rabbit secondary antibody conjugated with Cy3 (1:250; Jackson ImmunoResearch) and with DAPI (6-Diamidine-2-phenyl indole, dihydrochloride; 1:10 000; Sigma-Aldrich) in the blocking mixture for 1 h at RT. Sections were mounted with an anti-fading mixture (Fluoromount™ Aqueous Mounting Medium; Sigma-Aldrich).

Sections were observed under an epifluorescence microscope (Olympus Provis AX70) and photographed with a digital camera (XM10, Olympus). Thickness from the basal to the upper layer of corneal epithelium was measured with Image J (http://rsbweb.nih.gov/ij/). For the Pax6 staining, epithelial cells were counted using the "Cell Counter" function of ImageJ and the percentage of $Pax6^+$ cells was calculated. Five non-consecutive sections were used per animal and three measurements per section were performed: one at the centre and other two at each extreme.

Finally, for calculation of the portion of epithelial area stained for SPRR1B, a reconstruction of the cornea was performed from images at 200 magnifications. Five non-consecutive sections per cornea were used. We delimited the perimeter of the epithelium and we analyzed the percentage area with positive staining using the "Measure" function of ImageJ.

2.6. Evaluation of tear osmolarity and tear volume

Tear volume was measured with the phenol red thread test (PRT-TEST; Tianjin Jingming New Technological Development Co., Ltd. Tianjin, China). With jeweler forceps, the threads were placed in the lateral canthus for 30 s. Then, the threads were photographed under a microscope (Olympus Provis AX70) and wetting of the thread was

measured with Image J (http://rsbweb.nih.gov/ij/). Measurements were made without anaesthesia and at least 15 min before osmolarity measurements.

Tear film osmolarity was measured using the TearLab Osmolarity System (TearLab Corp San Diego, CA, USA) as previously described [9]. With a disposable test chip, tear was collected from the lateral canthus without anesthesia. Seconds later, a reading of the osmolarity was given in mOsml/l.

2.7. Cell culture

HCLE cells were plated in 12-well plates at a density of 10^5 cells per well. Cells were grown to confluence in keratinocyte serum free medium, supplemented with BPE and EGF as specified by the manufacturer (Gibco). At confluency, the medium was changed to DMEM/F12 containing 10% fetal bovine serum, 1% penicillin/streptomycin and 10 ng/ml of EGF, and cells were allowed to stratify for 1 week. Thereafter, the cells were washed 3 times with PBS, serum-starved for 1 h and incubated for 48 h with TNF-α (40 ng/ml), IL-1β (10 ng/ml). At 24 h, the medium was changed.

2.8. RNA isolation and real-time PCR

At 21 days post-HSCT or 3 and 10 days post-irradiation (in the case of *TBI* mice) mice were euthanized and the eyes were enucleated. Corneas were separated excluding the limbus and immediately frozen in liquid nitrogen. Four to five corneas per group were used. RNA was isolated using RNeasy kit (Qiagen) according to the manufacturer's instructions and using Eppendorf® micropestle (Sigma-Aldrich) for tissue disruption and homogenization. For RNA extraction from cultured cells, TRIzol® Reagent (Life Technologies) was used, according to the manufacturer's instructions. First-strand cDNA was synthesized from 200 ng of total RNA from mouse corneas or 1 μg of total RNA from cultured cells, using a reverse transcription kit (High Capacity Reverse Transcription Kit; Applied Biosystems, USA) according with the manufacturer's instructions. Quantitative RT-PCR (qPCR) was performed using SYBR® Green reagents (Power Sybr Green PCR Master Mix, Applied Biosystems, USA) with specific primers (Table 1). The following parameters were used: 10 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 1 min at 60 °C. All samples were normalized using glyceraldehyde-3-phoshate dehydrogenase (*GAPDH*) as housekeeping gene expression (Table 1). The comparative CT method was used for relative quantitation of *Tnfa*, *Il1b*, *Pax6* and *Sprr1b*, selecting the relative amount in *control* mice as the calibrator.

For detection of hMSCs, qualitative PCR was performed for detection of human *GAPDH* (Table 1) using GoTaq® Flexi DNA Polymerase (Promega). The amplification was initiated with 10 min of initial denaturation at 95 °C, followed by 40 cycles consisting in: 30 s at 95 °C, 30 s at 68 °C and 1 min at 72 °C. As positive control, cDNA from cultured hMSCs was used.

Table 1
Primer sequences for RT-PCR.

Gene	Primer sequence	Product size (bp)
TNFα	Fwd: TCAGCCTCTTCTCATTCCTG Rev: GGGTCTGGGCCATAGAACTGA	135
IL-1β	Fwd: CAACCAACAAGTGATATTCTCCAT Rev: GGGTGTGCCGTCTTTCATTA	127
Pax6	Fwd: AGTGAATGGGCGGAGTTATG Rev: ACTTGGACGGGAACTGACAC	131
SPRR1B	Fwd: GGCTCATCCATCTTTGAAGC Rev: AGGAACCTTGGTGTCACAGG	158
GAPDH	Fwd: ACTGGCATGGCCTTCCG Rev: CAGGCGGCACGTCAGATC	63
Human PAX6	Fwd: TGTCCAACGGATGTGTGAGT Rev: TTTCCCAAGCAAAGATGGAC	161
Human GAPDH	Fwd: GAGTCAACGGATTTGGTCGT Rev: GACAAGCTTCCCGTTCTCAG	185

2.9. Statistical analysis

The data are presented as mean ± standard deviation or median ± interquartile range. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software). Kolmogorov-Smirnov test was used to assess the normality of data distribution. Based on normality of the data distribution and the size of the sample, Kruskal-Wallis test was used for statistical comparison and Dunn's test as post-hoc test. A P value of < 0.05 was considered statistically significant.

3. Results

Balb/c mice were lethally irradiated as conditioning regimen and, 4 h later, received BM cells from MHC-mismatched C57BL/6 mice, complemented (*GVHD* group) or not (*BM* group) with spleen cells from the same donors. To determine if hMSC had a therapeutic effect in corneas affected by oGVHD, we injected these cells subconjunctivally on day 10 in some *GVHD* mice, enough time for GVHD to develop.

3.1. GVHD score and macroscopical ocular signs

Among days 4 and 7 post-HSCT, GVHD signs appeared in animals receiving splenic cells. GVHD score was similar in both *GVHD* and *GVHD + MSC* animals (3.67 and 3.36, respectively, at day 7 post-HSCT). Two weeks after transplantation, all mice ameliorated, but this improvement was more pronounced in *GVHD + MSC* mice, with a score of 1.45, than in *GVHD* mice, with a score of 2.42. On the third week, the *GVHD + MSC* maintained a lower *GVHD* score (2.27) compared to *GVHD* mice (3.67). Finally, on day 28 post-HSCT both *GVHD* and *GVHD + MSC* groups showed similar signs of systemic GVHD (6.08 and 5.82, respectively). No signs of systemic GVHD were found in the *BM* group but a slight weight loss on the first week after HSCT (Fig. 1A).

Ocular signs, previously reported by our group in this model [8,9], appeared from the first week in all animals transplanted with splenic cells. These signs included blepharospasm, erythemathous eyelids and loss of periocular fur. Ocular signs worsened on the third week and even more on the fourth week in untreated eyes (*GVHD* and *GVHD + MSC LE*), while treated eyes showed a much better aspect (Fig. 1B).

3.2. Immunohistological analysis

Four weeks after transplantation, we analyzed the therapeutic effect of hMSC on pathological features in mouse corneas. Six eyes per group were analyzed.

3.2.1. hMSCs prevent T lymphocyte infiltration

As previously demonstrated by our group, leukocyte infiltrates can be observed in cornea and limbus in this GVHD model [8]. We used an antibody against CD3, a surface protein characteristic of T lymphocytes, which are the main cell type responsible of GVHD damage. The *GVHD* group showed different levels of $CD3^+$ cell infiltration. In some corneas (2 of 6 corneas analyzed) we found the staining limited to the limbus while, in others, these cells reached the paracentral cornea and even the central cornea (4 of 6 corneas) (Fig. 2A–D). The amount of $CD3^+$ cells varied from small groups of cells (Fig. 2A and B) to massive infiltration (Fig. 2C and D). These cells were present mainly in the stroma, although it was frequent to observe positive cells close to the basal layer of the corneal epithelium. In contrast, corneas from mice treated with hMSC did not show any infiltration of $CD3^+$ cells (Fig. 2E and F). As for *GVHD + MSC LE* eyes (contralateral eyes), $CD3^+$ cells were scarcely observed and they were always limited to the limbus (data not shown). This result suggests a protective effect of hMSC against T lymphocyte infiltration.

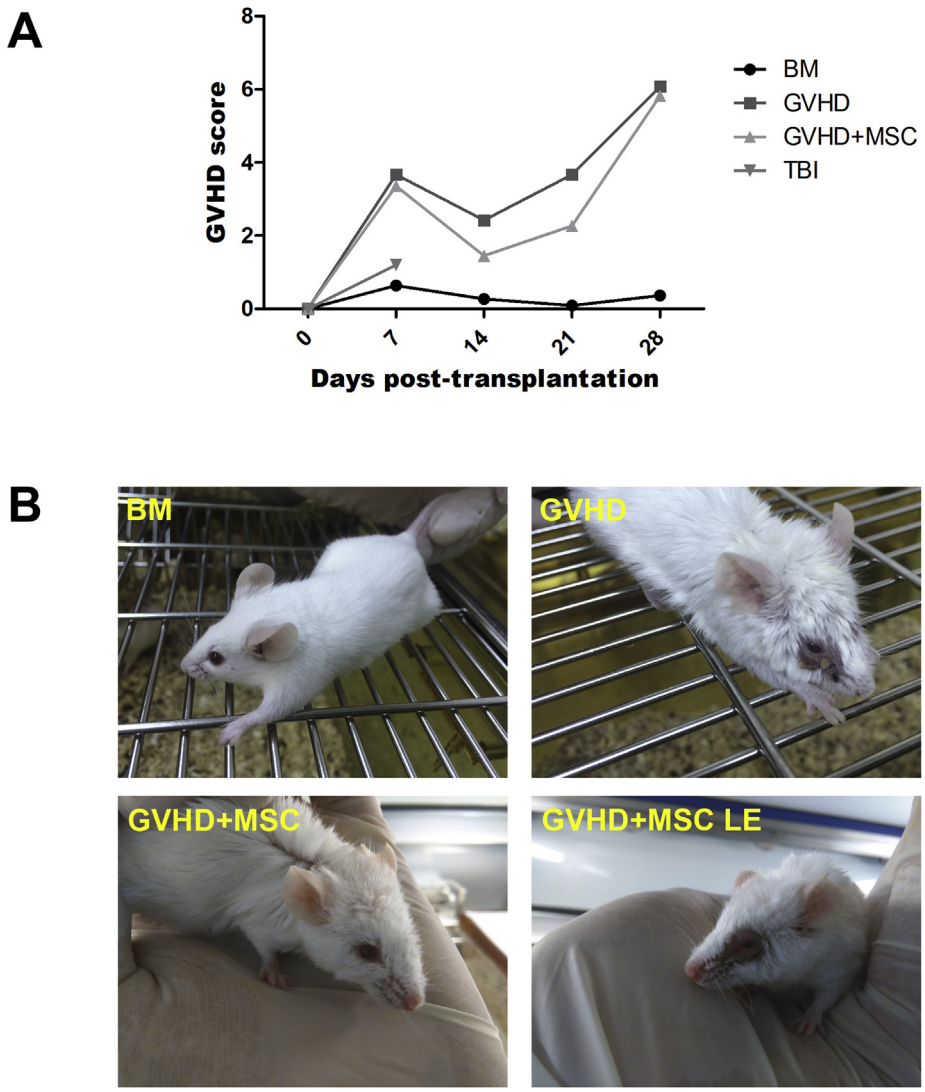

Fig. 1. Macroscopical signs of GVHD. (**A**) Evolution of clinical GVHD score in mice after HSCT. At day 28 post-HSCT, mice both treated and untreated exhibited similar clinical signs characteristic of systemic GVHD. In contrast, *BM* remained healthy until this day. (**B**) Representative images of transplanted mice at 28 days post-HSCT. Images of GVHD + MSC and GVHD + MSC LE eyes were taken from the same animal.

3.2.2. hMSCs reduce keratinization of the corneal epithelium

We evaluated pathological features in corneal epithelium. Our group has also shown in previous studies a decrease in the thickness of corneal epithelium [9]. Squamous metaplasia is a common pathologic process of the ocular surface that is mediated by inflammation [31,32]. We analyzed corneal epithelium thickness and also the presence of Pax6 in epithelial cells, since some authors have proposed that loss of this protein indicates keratinization [33,34].

Only *GVHD* corneas showed differences in epithelial thickness (33.61 ± 4.23 μm) compared to control corneas (43.34 ± 3.51 μm) (Fig. 3B). In contrast, *BM* corneas had similar values to the control (43.56 ± 3.26 μm). In treated animals, the corneas of treated eyes did not show differences with control, despite of showing a great reduction (36.90 ± 1.99 μm) and neither the corneas of the contralateral eyes (38.16 ± 1.22 μm) (Fig. 3B).

Calculating the percentage of Pax6+ nuclei in the epithelium, we only observed Pax6 loss in corneas of treated animals, both *GVHD + MSC* (45.36 ± 8.59%) and *GVHD + MSC LE* (30.92 ± 4.67%) (Fig. 3A and C). Corneas from *GVHD* animals exhibited values of Pax6 (86.65 ± 7.88%) similar to control (83.53 ± 2.30%), while *BM* corneas showed greater percentage (95.23 ± 0.85%). These results may suggest that the treatment was inducing keratinization of corneal epithelium. To test this hypothesis, we used antibodies against SPRR1B, a protein of the cornified envelope. No staining was found in control and *BM* corneas, while *GVHD* corneas showed keratinization foci with positive staining (Fig. 4A). In *GVHD + MSC* and *GVHD + MSC LE* the presence of SPRR1B was similar to control corneas and only in a few cases we found some superficial positive staining (Fig. 4B). We analyzed the percentage of the epithelial area with positive staining to compare between groups. In control corneas the stained area was the 0.09 (0.09–0.09) % (median and IQR) and *BM* corneas showed similar values (0.07 (0.03–0.08) %), corresponding with background staining. *GVHD* corneas showed a greater area stained for SPRR1B, with a median of 1.28 (0.34–4.32) % ($P < 0.05$ compared to *BM* group), while *GVHD + MSC* and *GVHD + MSC LE* showed 0.16 (0.05–1.85) % and 0.11 (0.06–1.15) %, respectively (Fig. 4C). Hence, we found that, although we observed a reduction in Pax6 staining, keratinization of corneas from MSC-treated mice was lower compared to untreated animals.

3.3. Tear osmolarity is not increased after treatment with hMSCs

At day 21 post-HSCT, treated mice improved their appearance compared to untreated mice, as reflected by the differences in GVHD

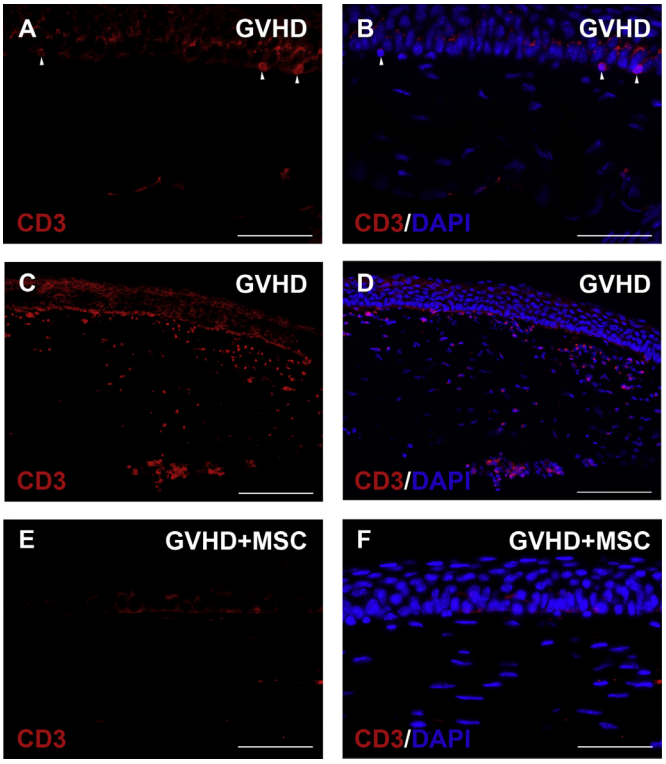

Fig. 2. Treatment with hMSCs abolished infiltration of T cells in the cornea. (**A-D**) Representative images of *GVHD* corneas, where presence of $CD3^+$ cells (red) is evident (arrowheads). Images **A** and **B** show a cornea with a moderate presence of $CD3^+$ cells, while images **C** and **D** show a cornea with massive infiltration. (**E, F**) Images of *GVHD + MSC* corneas; no $CD3^+$ where detected. Nuclei stained with DAPI. Scale bar: 50 μm (**A, B, E, F**); 100 μm (**C, D**).

score and the ocular signs. Previous observations from our group have shown that tear dysfunction is present in this model 3 weeks post-HSCT [9]. Hence, we analyzed the tear composition at 21 days post-HSCT to determine whether hMSC had an effect in dry eye signs (Fig. 5).

Tear osmolarity in *GVHD* and *GVHD + MSC LE* eyes (351 ± 25 mOsm/l and 350 ± 25 mOsm/l, respectively) was significantly higher ($P < 0.01$ and $P < 0.05$, respectively) than in *BM* eyes (326 ± 19 mOsm/l), while *GVHD + MSC* eyes (332 ± 25 mOsm/l) showed no differences compared to the *BM* group (Fig. 5).

The tear volume was lower in *GVHD* and *GVHD + MSC LE* eyes (2.38 ± 0.78 mm and 2.32 ± 0.75 mm, respectively) than in *BM* and *GVHD + MSC* eyes (2.88 ± 0.79 mm and 2.92 ± 0.98 mm, respectively). However, no significant differences were found between groups ($P = 0.07$) (Fig. 5).

3.4. hMSCs reduce the expression of inflammatory cytokines

Given the effects that tear dysfunction has in cornea (e.g., increase in cytokine expression and keratinization) [35,36], we wondered if the effects of hMSCs in tear osmolarity could lead to a reduction in cytokine expression. We isolated RNA from five corneas of each group (except for the *GVHD* group, where both corneas of five mice were used) and analyzed the expression of *Il1b* and *Tnfa*.

In general, there was no increase in *Il1b* gene expression, and there was even a decrease in *BM* corneas. Only a few corneas of the *GVHD* group showed a 5–6 fold increase (2 corneas from different animals out of a total of 10 corneas) (Fig. 6). However, expression of *Tnfa* was highly increased in all groups but *GVHD + MSC* corneas (Fig. 6). Even the contralateral eyes of these animals (*GVHD + MSC LE*) had high levels of the transcript. Surprisingly, *BM* corneas exhibited also this significant increase in cytokine expression.

We also analyzed the expression of *Pax6* and *Sprr1b*. *Pax6* expression was increased in all groups but, again, not in *GVHD + MSC*

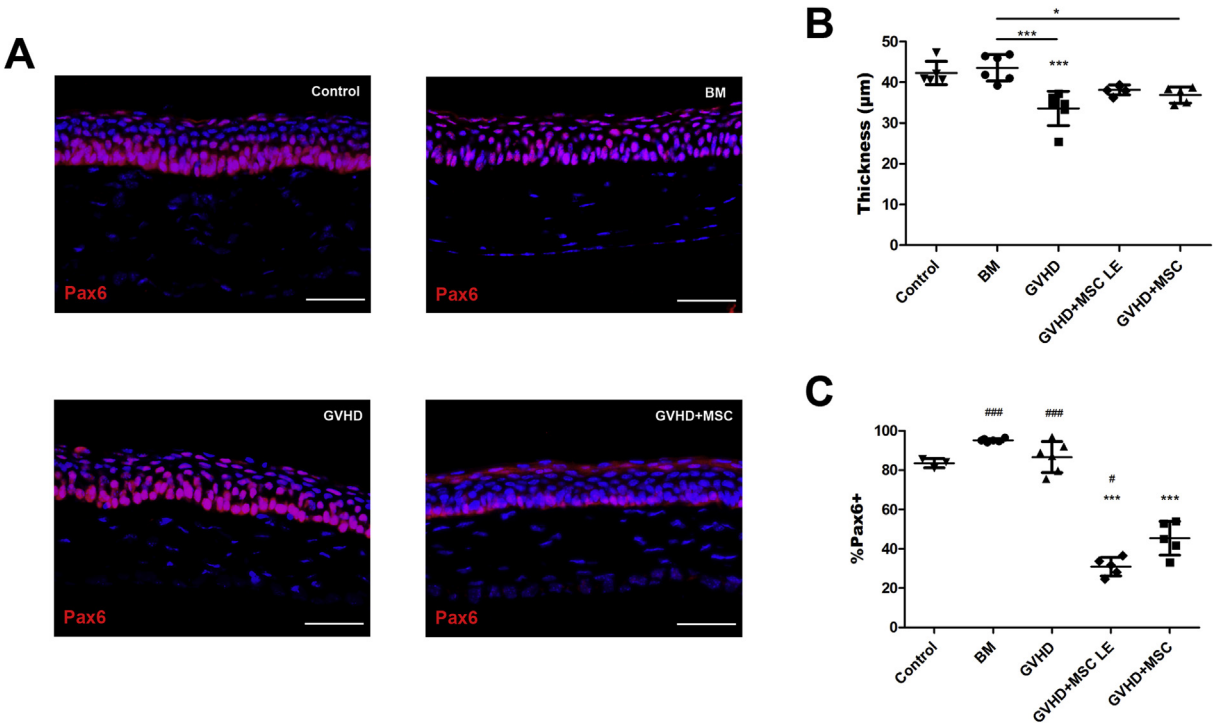

Fig. 3. Analysis of the presence of Pax6 and corneal epithelial thickness after treatment with hMSCs in the eye of mice with GVHD. (**A**) Immunofluorescent staining of nuclear Pax6 (red) in epithelium of *control*, *BM*, *GVHD* and *GVHD + MSC* corneas. Nuclei stained with DAPI. Scale bar: 50 μm (**B**) Measurement of epithelial thickness. Only *GVHD* corneas showed significant decrease of epithelial thickness compared to *control*. (**C**) Quantitative analysis of Pax6 staining expressed as percentage of $Pax6^+$ nuclei. Corneas from mice treated with hMSCs exhibited a great reduction of Pax6. $^*P < 0.05$ and $^{***}P < 0.001$ compared with *control*; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared with *GVHD + MSC*. Data are presented as mean ± standard deviation.

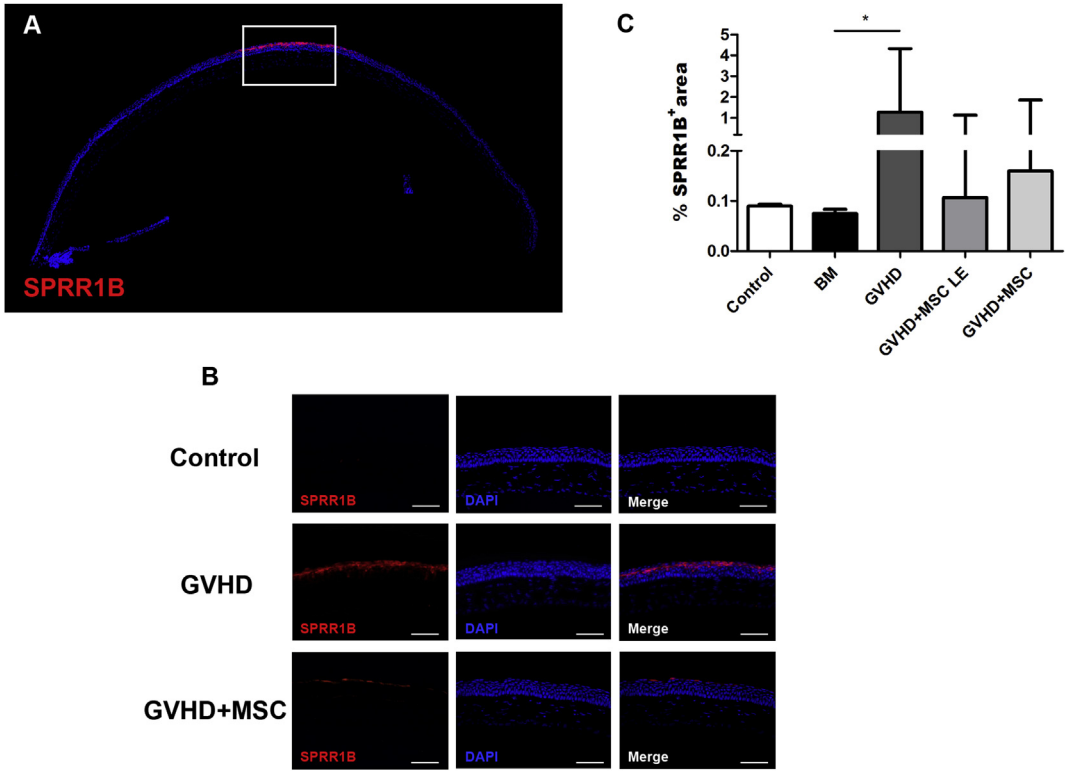

Fig. 4. The treatment with hMSCs decreases the presence of SPRR1B. (**A**) Reconstruction of a corneal section of the *GVHD* group, where positive staining for SPRR1B (red) can be seen in the central cornea. (**B**) Detail of corneas from *control*, *GVHD* and *GVHD + MSC* groups. (**C**) Quantitative analysis of the epithelial area expressed as percentage of the area with positive staining for SPRR1B. *GVHD* corneas exhibited a great increase of SPRR1B, while corneas from treated mice presented similar staining compared to *control*. *P < 0.05. Data are presented as median ± interquartile range.

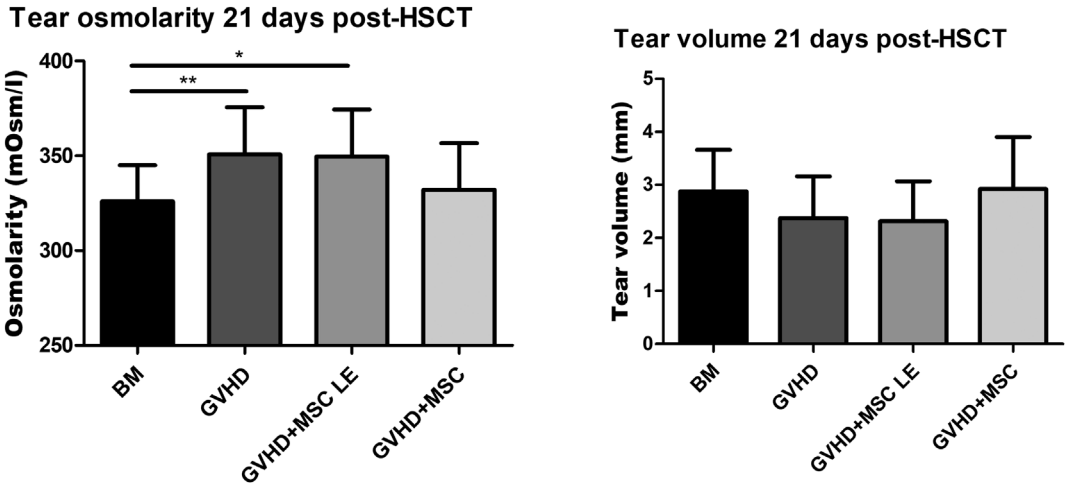

Fig. 5. Tear osmolarity and volume in mouse eyes at 21 days post-HSCT (11 days after hMSCs injection). Tear osmolarity was increased in untreated eyes (351 ± 25 mOsm/l in GVHD eyes and 350 ± 25 mOsm/l in GVHD + MSC LE eyes) compared to BM animals (326 ± 19 mOsm/l) but not in treated eyes (GVHD + MSC; 332 ± 25 mOsm/l). No significant differences (P = 0.07) were found in tear volume. *P < 0.05 and **P < 0.01. Data are presented as mean ± standard deviation.

corneas, where its expression was slightly reduced (Fig. 6). *Sprr1b* expression was reduced in those corneas where *Pax6* was increased. However, some *GVHD* corneas showed an increase in *Sprr1b* expression (2 out of a total of 10); the same corneas in which we observed an increase in *Il1b* (Fig. 6).

3.5. The hMSC did not engraft

To determine whether hMSC would engraft in the corneas of treated animals, we carried out PCR assay for human GAPDH in corneas of animals at 21 and 28 days post-transplantation. Results showed no amplification of *GAPDH* RNA in treated corneas (data not shown), indicating that the effects of hMSC are not explained by their engraftment in the cornea.

3.6. TNF-α does not induce an increase in PAX6 expression in vitro

Given the apparent correlation between the increase in gene expression of *Tnfa* and *Pax6*, we considered the possibility that this cytokine was inducing an increase in *Pax6* expression. To evaluate this

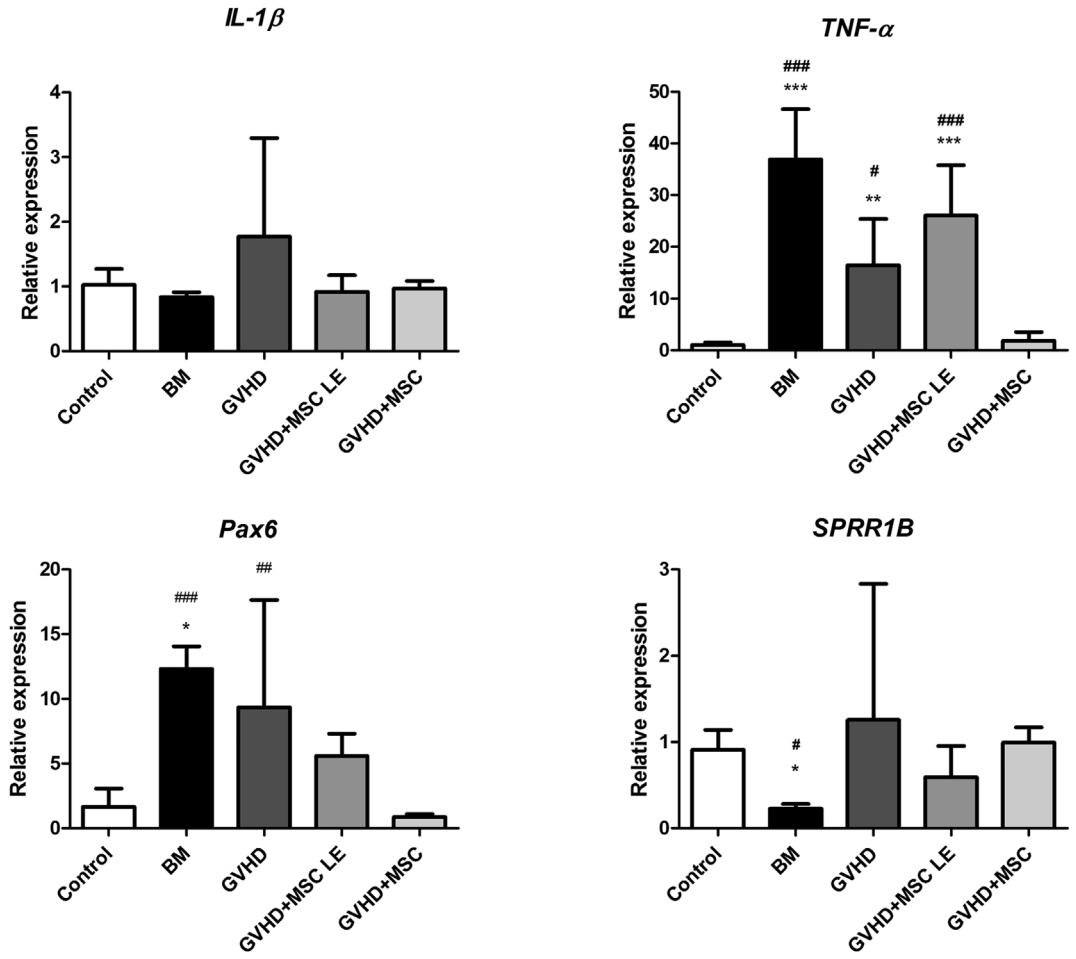

Fig. 6. Results of qPCR in cornea after treatment with hMSCs in the eye of mice with GVHD at 21 days post-HSCT. No changes were observed in *Il1b* except in some *GVHD* corneas (2 out of a total of 10). Only *GVHD + MSC* corneas showed levels of *Tnfa* and *Pax6* transcripts similar to *control*. *Sprr1b* gene expression was increased only when expression of *Il1b* was also elevated. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with *control*; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.001 compared with *GVHD + MSC*. Data are presented as mean ± standard deviation.

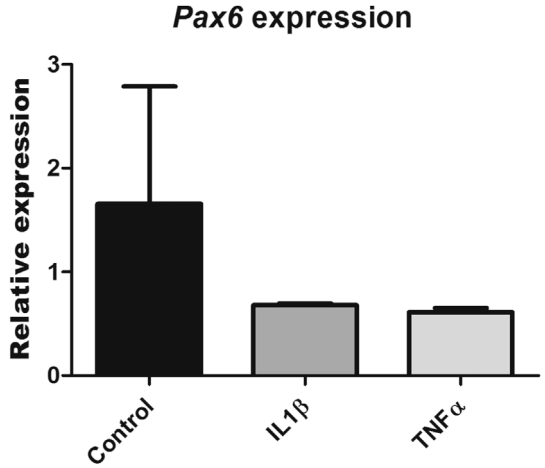

Fig. 7. Gene expression of *PAX6* in HCLE cells after treatment with IL-1β or TNF-α. Both cytokines decreased the expression of *PAX6*.

effect, we tested the effect of TNF-α and IL-1β on human corneal-limbal epithelial cells (HCLE cells) in vitro. *PAX6* expression did not increase and even decreased after addition of both cytokines, showing that the effect of TNF-α is not the cause of what we observe in mice (Fig. 7).

3.7. Total body irradiation induces the expression of Tnfa and Pax6 in mouse corneas

Since *Tnfa* and *Pax6* were increased in *BM* group in a similar way that in diseased animals, we wanted to know if total body irradiation could be the cause. To study the effects of irradiation on the cornea, we performed experiments with animals that received only total body irradiation (*TBI* group) and then we analyzed the gene expression after 3 and 10 days post-irradiation. We found that *Il1b* expression was decreased after irradiation. In contrast, *Tnfa* increased by day 3 and more intensively by day 10 (Fig. 8). *Pax6* expression increased 3 days after irradiation and decreased by day 10, but remaining 2.9 fold above the control levels. These results suggest that irradiation is the cause of *Tnfa* and *Pax6* increase in *GVHD* animals.

4. Discussion

Although not lethal, problems derived from oGVHD are one of the main causes of deterioration of quality of life in patients with GVHD [6,7]. To date, there is no effective treatment for this condition and the strategy is usually based in the use of tear substitutes and immunosuppressive therapies [2,37]. A worse outcome can be expected in patients who are refractory to steroids [13]. Administration of MSC as an immunomodulatory strategy has had good outcomes in corneal transplant, wound healing and dry eye models [18–24]. In the present study, we have demonstrated the protective effect of MSC in the cornea

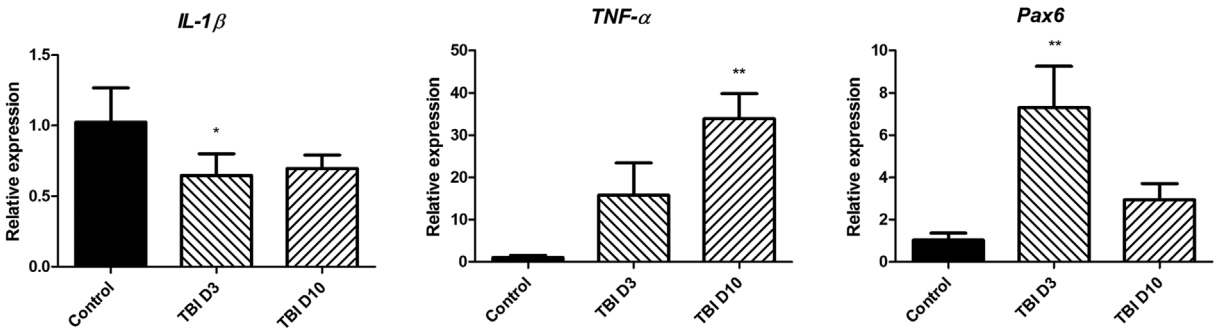

Fig. 8. Analysis of gene expression of *Il1b*, *Tnfa* and *Pax6* in cornea after total body irradiation. *Il1b* expression decreases after irradiation, while *Tnfa* and *Pax6* are increased. *P < 0.05 and **P < 0.01. Data are presented as mean ± standard deviation.

of mice using a well-established model of GVHD.

4.1. Systemic and ocular GVHD signs

The assessment of systemic GVHD was performed through a previously used score system [30]. Here, we could not perform a quantitative analysis as the individual parameters in this score system have a range of only 3 points (0–2) subjectively assigned, and we cannot consider these parameters as quantitative. However, we found obvious differences in the score of *GVHD* and *GVHD + MSC* mice at 14 and 21 days post-HSCT (Fig. 1A). A systemic effect of the locally applied treatment is improbable. To find a possible explanation, we have to pay attention to the parameters that differ between both groups. The parameters that showed evident differences were hunching, activity and fur texture. These parameters have one thing in common: they are common indicators of animal welfare. Hence, the most probable explanation is that the treatment ameliorated eye discomfort and that this is reflected in the mentioned parameters, all of them indicators of pain and distress. Ocular signs were much less evident in the treated eye, supporting the idea of an improvement in eye discomfort (Fig. 1B).

Tear film osmolarity, an important factor in corneal sensation [38,39], was not significantly increased in treated eyes, while it was in the untreated eyes (Fig. 5). An improvement in tear osmolarity values may reflect a reduction in discomfort.

4.2. hMSCs inhibit corneal inflammation in mice with oGVHD

Previous studies with mice have already demonstrated that immune cells invade the cornea in GVHD [8,11]. T cells have a critical role in GVHD and similar immunological diseases of the ocular surface, such as Sjögren Syndrome and other forms of dry eye. Inhibition of lymphocytic infiltration is essential, given the key role these cells have in corneal damage [31,37]. Our data demonstrate that subconjunctival injection of hMSCs protects the cornea from T cell invasion in oGVHD (Fig. 2). Moreover, contralateral eyes exhibited a great reduction in the number of $CD3^+$ cells in the cornea. This bilateral effect of hMSCs injection could be mediated by corneal nerves, whose intimate relationship with the immune system is well known [40].

The underlying mechanisms that lead to corneal damage in GVHD are poorly understood. We know that the cornea of mice with oGVHD is exposed to elements that may alter its milieu, like tear disturbances [9,10]. As demonstrated in previous studies, desiccating stress and tear hyperosmolarity induce an inflammatory response through the expression of cytokines like IL-1β and TNF-α [35]. Alteration of the corneal milieu through the action of proinflammatory factors disturbs the natural immune privilege of the cornea. In our experiments, untreated eyes (*GVHD* and *GVHD + MSC LE*) had increased osmolarity values, while hMSCs treated eyes did not (Fig. 5). How the hMSCs can achieve this event must be studied in the future. A possibility is that hMSCs exert a paracrine effect from the site of injection, helping to reduce the initial immune reaction and avoiding the damage to the lacrimal functional unit.

But conditioning regimens, such as irradiation, may also induce the release of cytokines in other locations, in a process known as "cytokine storm", which is considered the starting point of acute GVHD [1,36,41]. He et al. [42] have observed that conditioning regimens affect the cornea, reducing the corneal nerve density. Our mice did not show elevated levels of *Il1b* expression except in the very affected mice. In contrast, *Tnfa* expression was increased in all groups except in eyes treated with hMSCs (Fig. 6). Two conclusions can be extracted from this: the first is that subconjunctival injection of hMSCs was effective in reducing the expression of *Tnfa*; the second is that the corneas of *BM* mice are not completely healthy, since they show elevated levels of *Tnfa* expression.

The experiments with TBI mice demonstrate that the conditioning regimen is enough to increase *Tnfa* expression in the cornea. TNF-α is a potent proinflammatory cytokine, which has been related, among others roles, with the increase of leukocyte infiltration, via selectins' upregulation and endomucin downregulation in endothelial cells [43]. In our study, the presence of $CD3^+$ cells in the cornea of diseased mice could be facilitated by the increase in TNF-α provoked by irradiation. We speculate that the reduction in the expression of *Tnfa* in eyes treated with hMSCs may be a mechanism to reduce T-cell infiltration.

As no hMSCs were found in the cornea, the observed effect could be explained by the release of paracrine factors. Among the paracrine molecules produced by hMSC, TSG-6 has been found to exert an anti-inflammatory effect in a model of ocular damage [44], and it may have a role in the reduction of *Tnfa* gene expression that we observe.

The need for MSC cell engraftment into the corneas to achieve therapeutic effect is controversial. Mittal et al. [20] treated mice with MSCs by intravenous injection and they found cells homed in wounded but not in healthy corneas. Nevertheless, it was demonstrated that the positive results were also associated to the release of HGF. Engraftment of MSCs into injured corneal tissue has been also demonstrated in other injury models [45–47]. Moreover, in a previous manuscript [29] from our group we found engraftment of human cells in the corneal tissue of oGVHD animals, evaluated by immunohistochemistry and electron microscopy, with different sensitivity to the PCR technique for human *GADPH* detection used in the current work. Nevertheless, the potential contribution of the engrafted cells could be significantly lower than the paracrine effect, and the ability to detect MSC engraftment could be related to the sensitivity of the techniques used to assess it and the damage induced in the corneal tissue. In this regard, in the current manuscript a 20% reduction in the splenocytes administered (compared to our prior publication) may potentially reduce tissue damage and subsequent MSC attraction and lodging [15,48]. Other studies where the authors used subconjunctival injection of MSCs also showed positive results without detecting substantial corneal engraftment [18,24,26].

4.3. hMSCs inhibit corneal keratinization induced by oGVHD

Squamous metaplasia is an end-stage consequence of ocular inflammation that can progress to corneal opacification and vision loss [32]. Some authors, trying to find an early marker of this process, have pointed out to Pax6 loss [33,34]. In the present study, we did not find a relationship between Pax6 loss and the appearance of SPRR1B, a known marker of keratinization. While mice with oGVHD showed an increase in SPRR1B in corneal epithelium and normal values for Pax6, mice treated with hMSCs did not present SPRR1B but exhibited a decrease in Pax6 (Figs. 3 and 4). These results indicate that a reduction in Pax6 staining and expression is not always a good indicator of squamous metaplasia. Pax6 must have other roles in the present context, as discussed below.

The lower presence of SPRR1B in treated mice indicates a lower keratinization in corneas of this group. Moreover, the presence of SPRR1B is also low in the untreated eye of treated animals, suggesting a protective effect in the contralateral eye, which is in accordance with the lower infiltration of $CD3^+$ cells in these eyes. Squamous metaplasia is linked to inflammation, with $CD4^+$ T cells as the main effector cells and IL-β as a key inducer [31,32,49]. Hence, the reduction of corneal inflammation is crucial to avoid corneal keratinization. This is confirmed by the fact that only in corneas of the *GVHD* group we found an increase in *Il1b* gene expression and only in these corneas we found an increase in *Sprr1b* gene expression.

The loss of Pax6 in corneas from treated eyes and its increase in untreated animals is intriguing. Based on previous observations indicating that TNF-α increases the expression of *Pax6* in HeLa cells [50], we exposed HCLE cells to this cytokine. However, we found that, in these cells, TNF-α did not induce an increase in Pax6, so the presence of this cytokine does not explain the increase in Pax6 observed in mice (Fig. 7). On the other hand, the increase in *Pax6* expression in TBI mice suggests that irradiation is the cause of the increase observed in diseased mice and also in *BM* mice (Fig. 8). Pax6 regulates the expression of enzymes like ALDH3A1, with detoxification activity [51], so a possible explanation is that corneal cells produce more Pax6 as a defensive mechanism against irradiation-derived damage. The relationship between Pax6 and TNF-α could be the opposite, being Pax6 the factor that induces an increase in TNF-α. A previous study in mouse brain cells demonstrated that Pax6 binds to the promoter region of *Tnfa*, among other inflammatory factors [52]. A future study could examine if this occurs also in corneal epithelial cells.

In processes of re-epithelialization mediated by epithelial growth factor (EGF), Pax6 is downregulated [53]. In addition, an increase in Pax6 suppresses epithelial cell proliferation [54]. Moreover, in patients with Sjögren Syndrome, a reduction of EGF in tear has been observed [55] and patients with oGVHD show reduced levels of the EGF receptor (EGFR) in the conjunctiva [56]. We speculate that, if EGF has an inhibitory effect in *Pax6* expression, loss of EGF could result in an increase of Pax6. Interestingly, among the factors released by MSCs, EGF is included [57]. The release of EGF by hMSCs could have an effect of re-epithelialization that repair the corneal epithelium. As previously demonstrated by our group, apoptosis occurs in the ocular surface of mice with oGVHD, but not when these mice are treated with hMSCs [29]. Hence, a decrease in Pax6 expression may be a mechanism to repair the cell loss, by increasing cell proliferation. This could explain why there is no difference in epithelial thickness between control group and the eyes of treated mice (Fig. 3B).

5. Conclusions

In summary, in the present study we demonstrate that T lymphocyte infiltration and corneal epithelial disturbances occurs in this model of oGVHD. Also, we have demonstrated the effect of conditioning regimen in these processes. Finally, and most importantly, we have shown the therapeutic potential of hMSCs on corneal pathology derived from GVHD; the mechanism by which these cells exert this effect must be elucidated in the future.

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