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Purpose. The present study was designed to investigate the role of ocular surface glycocalyx and mucins in graft versus host disease (GVHD)-associated dry eye. The ameliorative effect of topical rebamipide, a mucin secretagogue, on GVHD-associated dry eye was also tested.

Methods. A mouse model of allogeneic transplantation was used to induce ocular GVHD with C57BL/6 as donors and B6D2F1 as recipient mice. Phenol red thread method and fluorescein staining was used to quantify tear secretion and corneal keratopathy. At 8 weeks after the allogeneic transplantation, corneas were harvested to perform glycocalyx staining and confocal microscopy. Goblet cell staining was performed using periodic acid Schiff's staining. Corneal and tear film levels of Mucin 1, 4, 16, 19, and 5AC were quantified using ELISA and real-time PCR. Rebamipide was applied topically twice daily to mice eyes.

Results. Allogeneic transplantation resulted in ocular GVHD-associated dry eye characterized by a significant decrease in tear film volume and the onset of corneal keratopathy. Ocular GVHD caused a significant decrease in the area and thickness of corneal glycocalyx. A significant decrease in the goblet cells was also noted. A significant decrease in mucin 4 and 5AC levels was also observed. Topical treatment with rebamipide partially attenuated ocular GVHD-mediated decrease in tear film volume and significantly reduced the severity of corneal keratopathy.

Conclusions. Ocular GVHD has detrimental impact on ocular surface glycocalyx and mucins. Rebamipide, a mucin secretagogue, partially prevents ocular GVHD-associated decrease in tear film and reduces the severity of corneal keratopathy.

Keywords: Mucins, Dry eye, Graft versus host disease, Glycocalyx, Rebamipide

Allogeneic hematopoietic stem cell transplantation is a successful treatment option for hematologic malignancies. However, graft versus host disease (GVHD) is a serious complication of hematopoietic stem cell transplantation and its incidence remains high in spite of the advances in human leukocytes antigens (HLA) matching. Depending upon the time of onset and clinical manifestations, GVHD is divided into acute and chronic phases. Acute GVHD primarily affects liver, skin, and intestine.[1–3] On the other hand, chronic GVHD has been shown to cause a high incidence of ocular complications.[4,5] As high as 60% to 90% of chronic GVHD patients suffer from ocular manifestations.[4,5] Ocular signs in chronic GVHD patients may be noticeable even before the other systemic symptoms.[6–8] Dry eye disease is one of the most frequent complications of ocular GVHD.[9,10] The dry eye disease in ocular GVHD patients is severe, resulting in symptoms of blurred vision, photophobia, redness, gritty sensation, and pain.[9,10] These symptoms cause significant visual discomfort, and reduce the overall quality of life of GVHD patients.[9,10] In absence of timely and appropriate treatment, dry eye disease in GVHD patients may progress to corneal keratopathy, ulceration, and visual impairment.[8,11]

The lacrimal functional unit, including ocular surface nerves, apical surface glycocalyx, lacrimal glands, meibomian glands, and a normal blinking response, all collectively contribute to the secretion and maintenance of a healthy tear film.[12] Tear film is critical for keeping the ocular surface hydrated and lubricated, thus preventing desiccation-induced damage to the ocular surface. Tear film comprises the mucoaqueous gel layer, which underlies but partially integrates into lipid layer.[13]

Mucins are high molecular weight glycoproteins made up of a protein core with extensive glycan N-acetyl galactosamine side chains. The heavy glycosylation imparts the mucins with hydrophilicity and a negative charge.[14–18] These structural features account for the two key physiologic functions of ocular surface mucins, which include repelling pathogens and keeping the ocular surface hydrated.[14–18] The mucins present on the ocular surface and in the tear film include membrane-bound mucins MUC1, MUC4, MUC16, and gel-forming secreted mucins, MUC19 and MUC5AC, respectively.[14–18] The membrane-bound mucins are expressed on the apical surface of corneal and conjunctival epithelial cells. The gel-forming mucin MUC5AC in the tear film is primarily secreted by the goblet cells.[19] Lacrimal gland acinar cells have been shown to express MUC7 transcript but the glycoprotein has not been detected in the tear film.[20] Tear film mucins keep the eye surface lubricated and entrap allergens and pathogens.[14–18] Patients with dry eye disease show reduced levels of mucins or an alteration in the degree of their glycosylation.[21–23] A significant aqueous deficit has been observed in the tears of GVHD patients suffering from

dry eye.[24] Multiple studies have shown that GVHD causes lacrimal gland fibrosis.[25–28] The effect of GVHD on ocular surface mucins has not been investigated. Therefore, the aim of the present study was to investigate the role of ocular surface mucins in GVHD-associated dry eye and to test the ameliorative effect of rebamipide, a mucin secretagogue, on GVHD-associated dry eye.

Methods

Allogeneic Bone Marrow Transplantation

The animal protocol was approved by Institutional Animal Care and Use Committee of Chapman University. All the animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A previously published mouse model of major histocompatibility (MHC) class I mismatch-induced ocular GVHD was used.[28] The B6D2F1 mice (The Jackson Laboratories, Bar Harbor, ME, USA) having a heterozygous MHC haplotype b/d were used as recipients and C57BL/6 mice (The Jackson Laboratories) having a homozygous haplotype b/b were used as donors. The bone marrow and spleen cells were harvested from 8-week-old donor female C57B6 mice. Ten-week-old female B6D2F1 recipient mice were exposed to a total body irradiation of 1100 cGy delivered in two equally divided doses 3 hours apart (RS 2000 X-ray Biological Irradiator; Rad Source Technologies, Buford, GA, USA). The irradiated B6D2F1 mice were then injected with 2×10^6 spleen cells and 5×10^6 bone marrow cells obtained from C57B6 mice by retro-orbital injection. The mice were housed in sterile cages, fed with diet gel (ClearH_2O, Portland, ME, USA) and received sulfatrim (0.672 mg/mL) in their drinking water for the first 14 days. At 8 weeks after the transplantation, animals were euthanized by CO_2 administration for the collection of ocular tissue.

The study design included three different groups of mice. (1) The control group (no transplant; $n = 6$) mice included age-matched B6D2F1 mice, which did not receive any bone marrow or spleen cell transplantation. (2) The ocular GVHD group ($n = 12$) included B6D2F1mice that received allogeneic bone marrow and spleen cell transplantation. (3) The rebamipide-treated group ($n = 6$) included B6D2F1mice that received allogeneic bone marrow and spleen cell transplantation and were treated with 2 μL topical ophthalmic drops of 2% rebamipide suspended in balanced salt solution (BSS) in left eye two times daily. The right eye of these mice received 2 μL topical ophthalmic drops of vehicle BSS two times daily. The 2% dose of rebamipide was selected based on the previously published studies.[29] The volume of ophthalmic application was also selected based on the previously published studies that use 2-μL drop administration per mouse eye.[30–32]

Tear Quantification

Tear secretion was quantified by phenol red thread test before the allogeneic transplantation and at weekly intervals after the transplantation. The phenol red impregnated thread (FCI Ophthalmics, Pembroke, MA, USA) was placed in the lower eyelid of mice on the temporal side for 1 minute. Upon wetting by tears, the phenol red thread changes color from yellow to red due to pH change. After 1 minute, the thread was removed and the length of the red color on the thread was measured. The length was converted to the volume by using a standard curve plotted by measuring the length of the phenol red thread wetted with a known volume of artificial tears.[31,32] Due to the small amount of tear film volume in the mouse eyes, the phenol red thread test typically requires longer time to obtain consistent values. Therefore, this study used 1-minute duration for phenol red thread test in mice as is also reported in previous studies.[33,34]

Fluorescein Staining

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). A 1-μL sterile solution of 0.1% fluorescein was applied to mouse eye and imaging was performed under a green fluorescent filter using stereomicroscope equipped with a digital camera. The captured corneal images were divided into four hypothetical quadrants for scoring the keratopathy using a previously published method.[35] Each quadrant was scored as follows: no staining = 0; slightly punctate staining less than 30 spots = 1; punctate staining more than 30 spots, but not diffuse = 2; diffuse staining but no positive plaque = 3; positive fluorescein plaque = 4. The scores of each quadrant were added to arrive at a final grade (total maximum possible score = 16).

Quantification of Glycocalyx-Stained Area and Thickness

The eyes were collected from euthanized animals at 8 weeks after the allogeneic transplantation and were fixed by immersing overnight in 4% paraformaldehyde. The corneas were isolated and blocked in 5% BSA for 20 minutes. Glycocalyx staining on the corneas was performed using 1.5 μg/mL solution of Alexa 488 conjugated wheat germ agglutinin lectin (Thermo Fisher Scientific, Hanover Park, IL, USA) for 20 minutes. Wheat germ agglutinin lectin binds to the N-acetylglucosamine and N-acetylneuraminic acid residues present on the ocular surface glycocalyx and has been used in multiple studies, including human patients to stain corneal glycocalyx.[36–38] The stained corneas were imaged using a confocal microscope. A total of four images were captured from each cornea. The quantification of glycocalyx stained area and thickness was performed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) in a blinded manner.

Goblet Cell Staining

The eyes along with eyelids were harvested from euthanized animals at 8 weeks after the allogeneic transplantation and processed for paraffin embedding. The 7-μM thin paraffin sections were cut and Periodic Acid Schiff's (PAS) staining was performed for goblet cells using a commercially available kit (Polysciences, Inc., Warrington, PA, USA). The stained sections were imaged at ×100 magnification using a brightfield microscope (Keyence corporation of America, Itasca, IL, USA).

ELISA Quantification of Mucin 1, 4, and 16

At 8 weeks after the allogeneic transplantation, animals were euthanized by CO_2 administration. The eyeballs were collected and the corneas were separated. The corneas were homogenized in RIPA buffer containing protease inhibitor (Pierce, Thermo Fisher Scientific, Hanover Park, IL, USA). The total protein in the corneal homogenates was quantified by BCA method using a commercially available kit (Pierce, Thermo Fisher Scientific). The Muc1, 4, and 16 levels were quantified in the corneal protein lysates using commercially available ELISA kits (LSBio, Seattle, WA, USA). The mucin levels were normalized for the milligram of total protein in the corneal lysates.

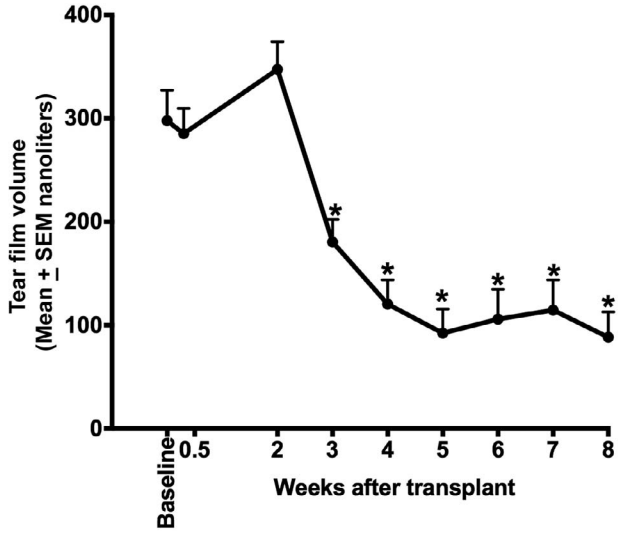

FIGURE 1. Tear film volume in mice before (baseline) and at various time points after allogeneic bone marrow and spleen cell transplantation. A significant (*$P < 0.05$ compared with baseline) decrease in tear film volume was observed at 3 weeks after allogeneic transplantation and it remained significantly low for the tested duration of 8 weeks.

ELISA Quantification of Mucin 5AC

The Muc5ac levels were quantified in the tears collected from mice at 8 weeks after the allogeneic transplantation. For tear collection, mice were lightly anesthetized with isoflurane. A 1 µL solution of 1× PBS containing 0.1% BSA was placed on each eye of the mouse and then collected back by using a Drummond microcapillary tube. The 1 µL collected from each eye was pooled and added to 8 µL of BSS solution. The tears were stored in –80°C for quantification of Muc5ac using a commercially available ELISA kit (LSBio, Seattle, WA, USA).

Gene Expression Quantification of Mucin 1, 4, 16, and 19

Corneas were harvested from animals at 8 weeks after transplantation as described above. The mRNA was extracted from the corneas using the RNeasy Mini kit (RNeasy kit; Qiagen Inc., Valencia, CA, USA). The mRNA was immediately reverse transcribed to cDNA using a commercially available kit (Superscript III First-strand synthesis; Thermo Fisher Scientific) for complementary (c)DNA synthesis. The cDNA was used to quantify Muc1, Muc4, Muc16, and Muc19 gene expressions using real-time PCR. A 20-µL reaction mixture containing 2 µL of cDNA, 2 µL of forward primer (200 nM), 2 µL of reverse primer (200 nM), and 10 µL of 2× SYBR green super mix was run at a universal cycle (95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 55°C for 60 seconds) in a thermocycler (Biorad CFX thermocycler; Bio-Rad Laboratories, Hercules, CA, USA). β-actin was used as the housekeeping gene. The relative change in gene expression was calculated using $^{\Delta\Delta}$Ct method.

Statistical Analysis

The data are presented as mean ± SEM. The data were tested for normal distribution using D'Agostino-Pearson omnibus normality test. One-way ANOVA followed by Dunnett's and Duncan's test was used to analyze time-dependent changes in tear film volume for Figure 1 and corneal keratopathy score for Figure 7B, respectively. The data presented in Figures 2 to 6 for comparing control and allogeneic transplantation groups were analyzed using unpaired *t*-test. Two-way ANOVA was used for data analysis of tear film volume presented in Figure 7A.

RESULTS

Effect of Ocular GVHD on Tear Film Volume and Corneal Keratopathy

The present study used MHC mismatched allogeneic transplantation mouse model that has been shown to develop ocular GVHD.[28] Our results further confirm that this mouse model of allogeneic transplant results in significant manifestations of dry eye due to ocular GVHD as is evident from a decrease in tear film volume and appearance of corneal keratopathy. Figure 1 shows a baseline mean ± SEM tear film volume of 300 ± 30 nL in the mice prior to the allogeneic transplantation. After the bone marrow and spleen cell transplantation, a statistically significant 3-fold decrease in tear film volume was noted starting at 3 weeks and this decrease persisted until the tested time point of 8 weeks ($P < 0.05$ compared with baseline). The observed 2-week delay in the onset of tear film decrease is anticipated because immune-mediated damage to the lacrimal

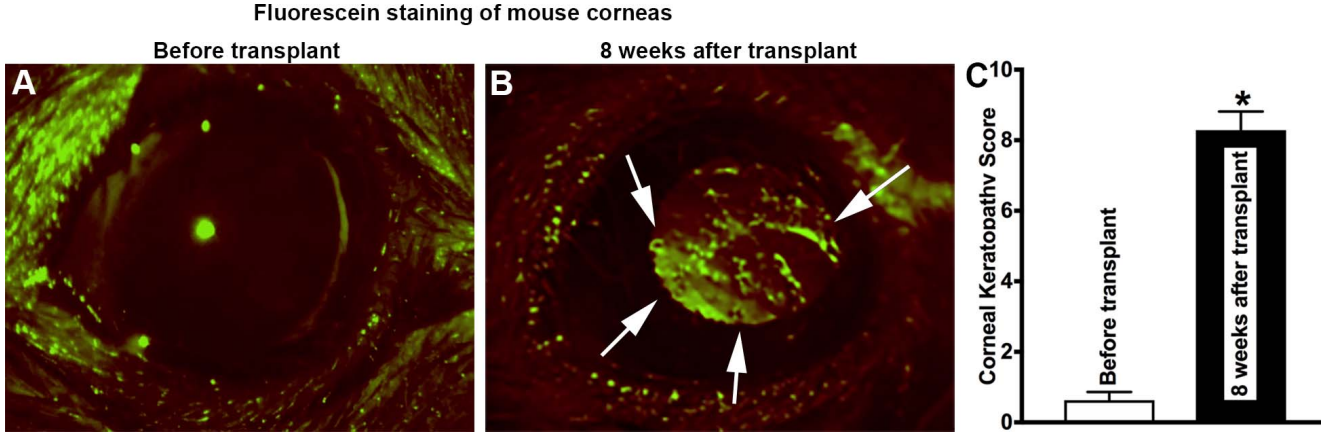

FIGURE 2. Representative fluorescein-stained images of mouse corneas before (**A**) and at 8 weeks (**B**) after allogeneic bone marrow and spleen cell transplantation. Quantification of fluorescein staining (**C**) showed significant (*$P < 0.05$ compared with before transplantation) corneal keratopathy at 8 weeks after allogeneic transplantation.

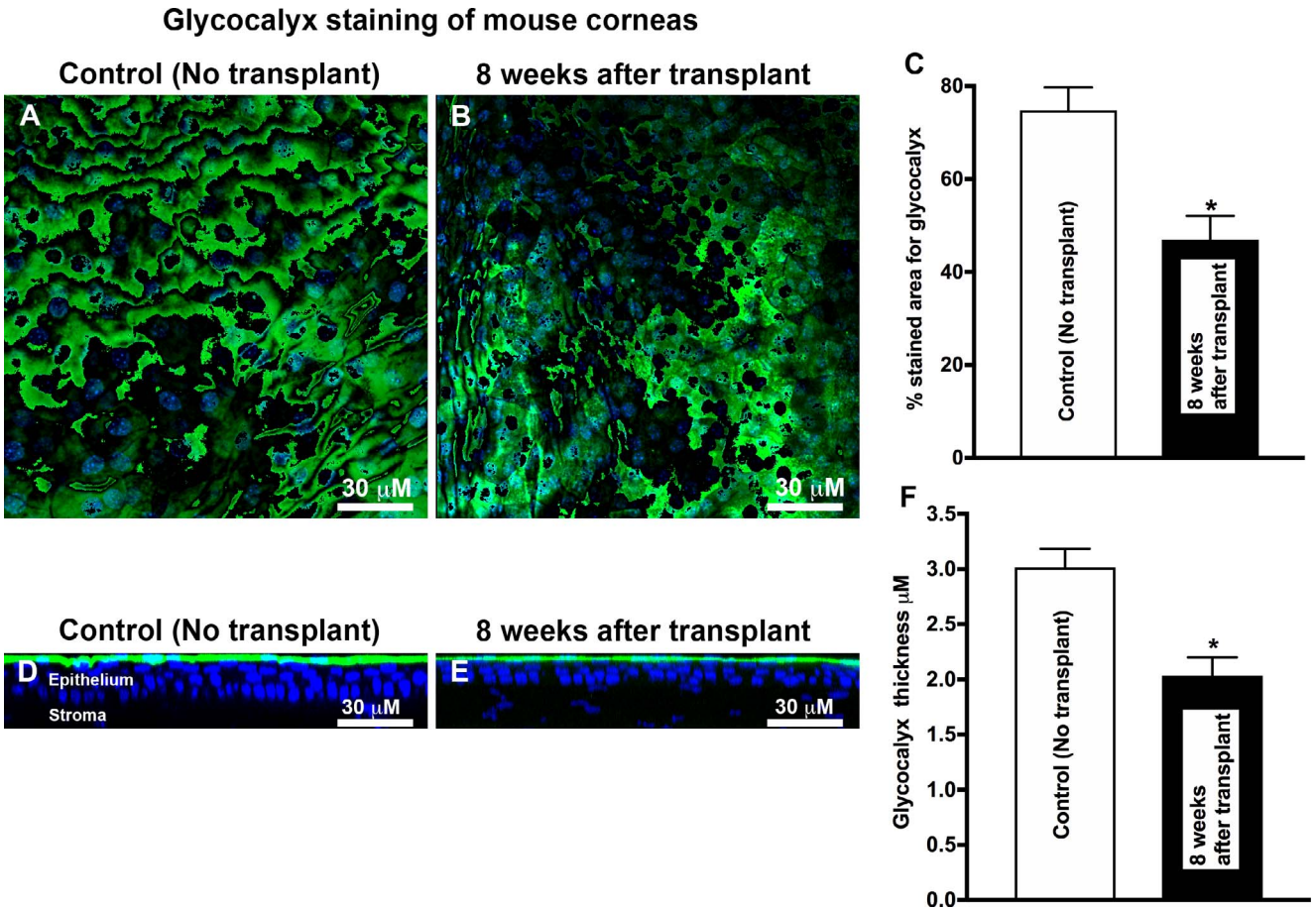

FIGURE 3. Representative confocal Z stacks images of top (**A**, **B**) and orthogonal (**D**, **E**) view of mouse corneas stained for glycocalyx (*green*) using wheat germ agglutinin. Nuclei are stained *blue*. Panel **A** and **B** is top view of corneas obtained from control mice that did not receive any transplantation and mice at 8 weeks after allogeneic bone marrow and spleen cell transplantation. Quantification of percent stained area (**C**) shows a significant decrease (*$P < 0.05$ compared with control mice that received no transplantation) in the glycocalyx in mice corneas at 8 weeks after allogeneic transplantation. Panel **D** and **E** is orthogonal view of corneas obtained from control mice that did not receive any transplantation and mice at 8 weeks after allogeneic transplantation. Quantification (**F**) shows a significant decrease (*$P < 0.05$ compared with control mice that received no transplantation) in the glycocalyx thickness in mice corneas at 8 weeks after allogeneic transplantation. Area and thickness quantifications were calculated using 16 different images each of control mice ($n = 4$) and mice that received allogeneic transplantation ($n = 4$).

functional unit is expected to precede prior to a decrease in tear film volume becomes apparent.

Figure 2 shows a representative fluorescein stained image of a mouse cornea before (Fig. 2A) and at 8 weeks after the allogeneic bone marrow and spleen cell transplantation (Fig. 2B). As can be seen in Figure 2B, the corneas of mice that underwent allogeneic transplant showed significant punctate and plaque staining. The scoring of fluorescein-stained corneal images was performed in a blinded manner using a previously described method.[24] The graph in Figure 2C shows that the corneas of mice that received allogeneic transplantation had a mean fluorescein staining score of 8 ± 0.5 ($P < 0.05$ compared with before transplantation) suggesting that ocular GVHD caused a moderate-to-severe degree of corneal keratopathy.

Effect of Ocular GVHD on Corneal Glycocalyx and Goblet Cells

Corneal epithelial cells express three different types of membrane-tethered mucins on their apical surface. These mucins together with galectin 3 form a continuous network of glycocalyx. Wheat germ agglutinin lectin binds to the sialic acid residues present on these mucins and has been previously used to stain the corneal glycocalyx.[38] Figure 3 shows the top and orthogonal projection confocal images of the mouse corneas stained for glycocalyx using Alexa 488 conjugated wheat germ agglutinin. The top view of confocal z stack images shows a dense and uniformly distributed glycocalyx staining in the corneas of control mice that did not receive any transplantation (Fig. 3A). On the other hand, corneal glycocalyx was sparse and patchy in the corneas obtained from mice at 8 weeks after they received allogeneic bone marrow and spleen cell transplantation (Fig. 3B). The glycocalyx-stained area was quantified as a percentage of the total area using binary image analysis of 16 images. Four images were collected from each cornea obtained from control mice ($n = 4$) and the mice that received allogeneic transplantation ($n = 4$). As is evident from binary quantification data presented in Figure 3C, a significant decrease of 37 ± 9% in glycocalyx-stained area was observed in the mice corneas that received allogeneic transplantation compared with the control mice without any transplantation ($P < 0.05$). Figures 3D and 3E show the orthogonal projection confocal Z stack images of glycocalyx-stained corneas. A significant decrease in the glycocalyx thickness was observed in the corneas obtained from mice that received allogeneic transplantation (Fig. 3D) compared with control corneas obtained from mice without any transplantation (Fig. 3E). Glycocalyx thickness was also

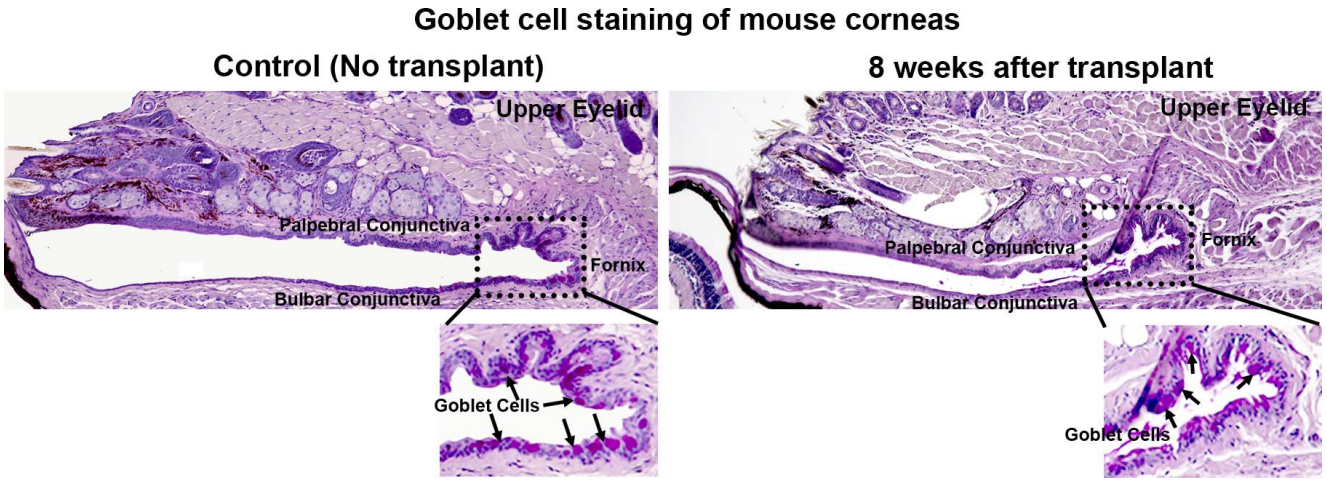

FIGURE 4. Representative images showing PAS-stained goblet cells in the tissue sections obtained from control mice (no transplant) and mice at 8 weeks after allogeneic bone marrow and spleen cell transplantation.

quantified in 16 images each obtained from corneas of control mice ($n = 4$) and from the corneas of mice that received allogeneic transplantation ($n = 4$). Figure 3F shows a mean decrease of 33 ± 6.6% in the corneal glycocalyx thickness in the mice that received allogeneic transplantation compared with control mice ($P < 0.05$).

Figure 4 shows a representative image of PAS-stained goblet cells in the eyelids of control mice and mice that underwent allogeneic bone marrow and spleen cell transplant. It is apparent from the staining that allogeneic bone marrow and spleen cell transplantation–mediated ocular GVHD caused a notable decrease in the number of goblet cells. It can also be noted that the morphology and mucin content of goblet cells has also been altered by the ocular GVHD in mice that received allogeneic bone marrow and spleen cell transplant as compared with the control mice that did not receive any transplantation.

Effect of Ocular GVHD on Mucins

We further investigated the effect of allogeneic bone marrow and spleen cell transplantation–associated ocular GVHD on membrane-bound Muc1, 4, and 16 mucins using corneal homogenates and on secreted Muc5ac in tear film. Figure 5A shows a reduction in Muc1 levels in the corneal homogenates obtained from mice at 8 weeks after allogeneic bone marrow and spleen cell transplantation (1.6 ± 0.7 ng/mg protein) as compared with the levels in the control corneal homogenates obtained from mice that did not receive any transplantation (0.8 ± 0.4 ng/mg protein) but the difference was not statistically significant. A statistically significant ($P < 0.05$) decrease in corneal homogenate levels of Muc4 (Fig. 5B) and tear film levels of Muc5ac (Fig. 5D) was also observed in the mice that underwent allogeneic transplant as compared with the control mice. On the other hand, a slight increase in Muc16 was observed (Fig. 5C) in the corneal homogenates of transplanted mice compared with control mice.

To test the effect of ocular GVHD on mucin gene expression, mRNA levels were quantified in the corneas of control mice and in the cornea obtained from mice at 8 weeks after the allogeneic transplant. A statistically significant ($P < 0.05$) 2.13 ± 0.35-fold increase in Muc1 gene expression was observed in the corneas obtained from mice that received allogeneic transplantation compared with control mice that did not receive any transplantation (Fig. 6A). A 1.25 ± 0.14-fold change in Muc4 mRNA levels (Fig. 6B), a 2.44 ± 0.7-fold change in Muc16 mRNA (Fig. 6C), and 0.265 ± 1.7 change in Muc19 mRNA (Fig. 6D) levels was observed in the corneas obtained from mice that received allogeneic transplantation compared with control mice that did not receive any transplantation. However, these changes in Muc4, 16, and 19 mRNA between control and GVHD mice were not statistically significant.

Effect of Topical Rebamipide on Ocular GVHD-Mediated Changes in Tear Film and Corneal Keratopathy

Last, we tested the effect of rebamipide, a mucin secretagogue, on ocular GVHD-mediated decrease in tear film volume and

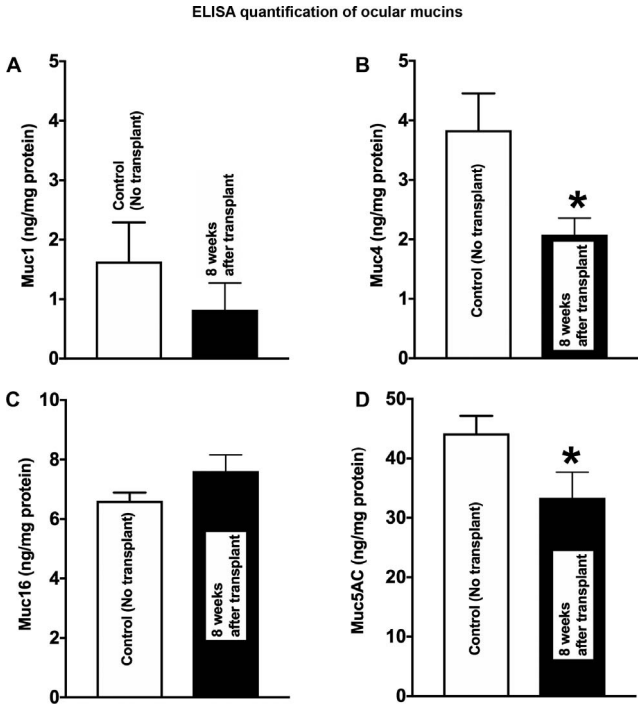

FIGURE 5. ELISA quantification of mucin 1 (**A**), mucin 4 (**B**), mucin 16 (**C**), and mucin 5AC (**D**) in the corneal homogenates and tears obtained from control mice (no transplant) and mice at 8 weeks after allogeneic bone marrow and spleen cell transplantation. A decrease in mucin 4 (*$P < 0.05$) and mucin 5AC (*$P < 0.05$) was observed compared with the levels in control mice that received no allogeneic transplantation.

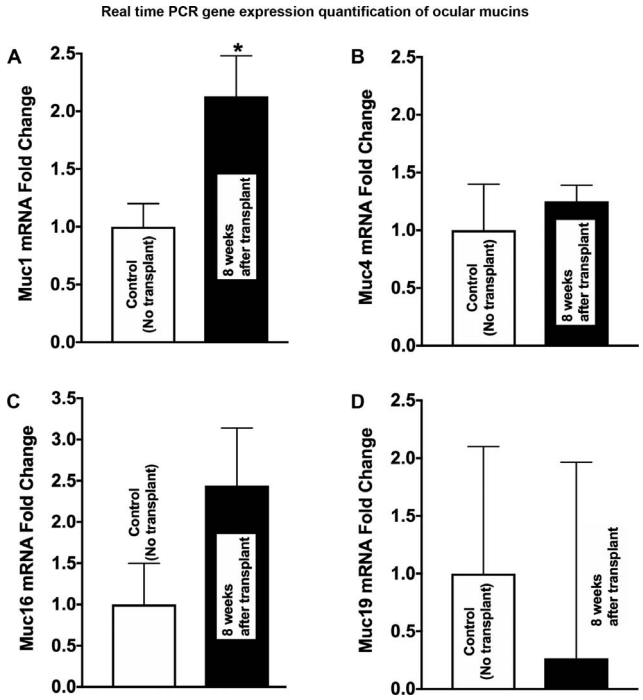

FIGURE 6. Gene expression quantification of mucin 1 (**A**), mucin 4 (**B**), mucin 16 (**C**), and mucin 19 (**D**) in the corneal homogenates obtained from control mice (no transplant) and mice at 8 weeks after allogeneic bone marrow and spleen cell transplantation. A significant increase in mucin1 gene expression (*$P < 0.05$) was observed compared with the levels in control mice that received no allogeneic transplantation.

cornel keratopathy. As can be seen from Figure 7A, twice daily topical ophthalmic application of rebamipide attenuated ocular GVHD-mediated decrease in tear film volume. The results were statistically significant ($P < 0.05$) at weeks 3 and 4 compared with the GVHD mice who received allogeneic transplantation but did not receive any eye drops. The BSS was used as a vehicle for compounding rebamipide. Therefore, we also tested the effect of topical ophthalmic application of BSS as vehicle but BSS application in GVHD mice had no notable effect on the tear film volume compared with untreated (no eye drops) control GVHD mice. Further, rebamipide application also significantly ($P < 0.05$) mitigated ocular GVHD-mediated corneal keratopathy (Fig. 7B). Rebamipide-treated GVHD mice showed a mean corneal keratopathy score of 3 ± 0.25 compared with a score of 8 ± 0.5 for the untreated (no eye drops) GVHD mice (Fig. 7B). It is interesting to note though that keeping the ocular surface hydrated by BSS vehicle application also partly attenuated corneal keratopathy. BSS-treated GVHD mice showed a mean corneal keratopathy score of 5 ± 0.5 compared with a score of 8 for the untreated (no eye drops) GVHD mice (Fig. 7B).

DISCUSSION

The apical surface of the corneal and conjunctival epithelium is covered with glycocalyx, a thin layer of glycoproteins largely composed of membrane-tethered mucins and galectin-3.[39–41] The glycocalyx forms a boundary between the ocular surface epithelium and the tear film. Glycocalyx serves to protect the cells against mechanical and chemical damage. An intact glycocalyx is also essential to reduce the friction during blinking and to keep the ocular surface hydrated.[39–41] We used fluorescent wheat germ agglutinin labeling and whole-cornea mount three-dimensional confocal microscopy to visualize glycocalyx on the corneas of GVHD mice. Wheat germ agglutinin binds to N-acetyl-d-glucosamine and sialic acid side chains of the membrane-tethered mucins and has been used to specifically label, visualize, and quantify glycocalyx in the cornea and vascular endothelium.[36–38] Our data demonstrate a significant decrease in the area and thickness of ocular surface glycocalyx in mice that received allogeneic bone marrow and spleen cell transplantation suggesting that GVHD has a detrimental effect on the ocular surface glycocalyx.

Membrane-tethered mucins are an integral component of glycocalyx. Thus, we further examined the effect of GVHD on corneal epithelial membrane-tethered mucins. Our results demonstrate that GVHD caused a significant decrease in protein levels of membrane-tethered Muc4 but did not cause any notable change in protein levels of membrane-tethered Muc1 or Muc16. Interestingly, a significant increase in Muc1 gene expression was observed in the corneas of GVHD mice, which can possibly be a compensatory response to partially circumvent the GVHD-mediated damage to the ocular surface glycocalyx. The ocular surface membrane-tethered mucins are heavily glycosylated and sialylated. These glycans, besides mucins, constitute an important component of the ocular surface glycocalyx. Studies have demonstrated a significant

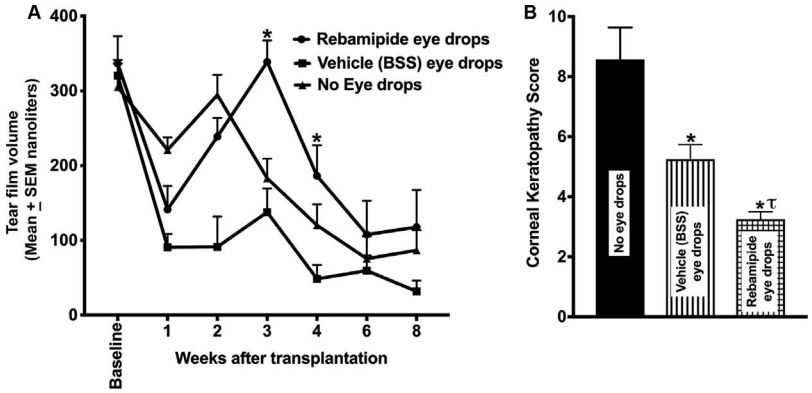

FIGURE 7. Effect of rebamipide ophthalmic drops on allogeneic bone marrow and spleen cell transplantation-mediated decrease in tear film volume (**A**) and corneal keratopathy score (**B**). Rebamipide attenuated allogeneic transplantation mediated decrease in tear film volume and the results were statistically significant (*$P < 0.05$) at 3 and 4 weeks after the allogeneic transplantation as compared with mice that also received the allogeneic transplantation but were either treated with BSS vehicle or did not receive any eye drops. Rebamipide treatment also significantly decreased the corneal keratopathy score (*$P < 0.05$ compared with no eye drop–treated mice; τ$P < 0.05$ compared with BSS vehicle). Animals that received BSS vehicle alone also showed significantly (*$P < 0.05$) lower corneal keratopathy score compared with mice that received no eye drops.

decrease in the glycosylation and sialylation of mucins in dry eye disease, keratinization, and contact lens wearers.[23,40,42] Lectin staining used in this study primarily binds to glycan carbohydrate part of the glycocalyx.[36–38] Because we observed a decrease solely in Muc4 while noting a significant decrease in the area and thickness of glycocalyx, our data raise the possibility that the observed changes in glycocalyx could possibly be due to a reduction in glycosylation of mucins.

Although detection of Muc16 in mouse cornea was not an objective of this study but our results show the presence of Muc16 in the mouse corneal lysates using ELISA and real-time PCR. The validity of our data was further confirmed by using mouse brain tissue as a negative control (data not shown). Our data are in contrast to two previously published papers that did not detect Muc16 in the mouse cornea using immunostaining.[43,44] Different levels of detection sensitivity of the techniques used in our study (ELISA and real-time PCR) as compared with immunostaining detection of Muc16 in formaldehyde-fixed paraffin sections used in the previous studies may explain this apparent discrepancy.[43,44]

Besides membrane-tethered mucins, tear film also contains soluble mucins. Goblet cells are the primary source of large gel-forming mucin Muc5AC, which is secreted into the tear film.[45,46] The results of the present study demonstrate that GVHD has a detrimental effect on goblet cells because a decrease in the number of goblet cells was observed in the tissue sections obtained from mice suffering from GVHD due to allogeneic transplantation. The histology observations are further supported by the ELISA quantification data showing a significant decrease in the tear film levels of Muc5AC in GVHD mice. Alterations in ocular surface mucins and glycocalyx has been previously reported in nonautoimmune dry eye and dry eye due to Sjogren's disease.[21,23,39,40] To the best of our knowledge, this is the first study to demonstrate that GVHD causes a damage to the ocular surface glycocalyx and alters ocular surface mucins.

In this study, we used an allogeneic MHC heterozygous-mismatch hematopoietic transplant mouse model to induce ocular GVHD. Our data demonstrate that this mouse model develops ocular GVHD-associated dry eye as demonstrated by a significant decrease in tear film and the corneal keratopathy. Previous studies have shown the development of ocular GVHD in this mouse model and support the results of the present study.[28] Using this mouse model, Hassan et al.[28] have demonstrated that GVHD has a detrimental effect on the lacrimal gland. Studies using MHC-matched allogeneic hematopoietic transplant mouse have also shown lacrimal gland damage in ocular GVHD.[47–49] However, our data are the first to demonstrate that besides the lacrimal gland, GVHD also causes damage to the ocular surface glycocalyx.

Rebamipide, an amino acid analog of 2 (1H)-quinolinone, has long been used for the treatment of gastric ulcers.[50,51] The ophthalmic formulation of rebamipide has recently been launched for the treatment of dry eye in Japan.[52] Rebamipide has been shown to stimulate gastric mucosal prostaglandin production, increase gastric mucus synthesis, and scavenge reactive oxygen radicals.[53–56] Recent studies have shown that rebamipide increases MUC1, MUC4, and MUC16 synthesis in stratified cultures of human corneal epithelial cells.[57,58] In vivo administration of rebamipide has been demonstrated to have an ameliorative effect in mouse model of Sjogren's syndrome, superoxide dismutase knockout mice, and rabbit model of dry eye.[29,59,60] Given the beneficial effects of rebamipide on mucous layer and dry eye, we tested the effect of topical administration of rebamipide in GVHD-associated dry eye mouse model. Our data demonstrate that topical rebamipide administration provided significant protection against GVHD-associated dry eye as indicated by the sustenance of tear film and a notable decrease in corneal keratopathy score. Interestingly, topical administration of BSS vehicle alone also had some ameliorative effect suggesting that keeping the ocular surface hydrated can partially rescue GVHD-associated corneal keratopathy.

In summary, our results demonstrate that allogeneic transplantation-associated ocular GVHD can have significant detrimental effects on ocular surface glycocalyx and ocular surface mucins. Further, modulation of ocular surface mucins by rebamipide, a mucin secretagogue, can partially prevent ocular GVHD-associated decrease in tear film and reduce the severity of corneal keratopathy.

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