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/6J 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
GVHD-Associated Dry Eye and Ocular Surface Mucins

Multiple studies have shown that GVHD causes lacrimal gland fibrosis. 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. 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 C57Bl6 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 x 10^6 spleen cells and 5 x 10^6 bone marrow cells obtained from C57Bl6 mice by retro-orbital injection. The mice were housed in sterile cages, fed with diet gel (ClearH2O, Portland, ME, USA) and received sulfafrim (0.672 mg/mL) in their drinking water for the first 14 days. At 8 weeks after the transplantation, animals were euthanized by CO2 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 B6D2F1 mice that received allogeneic bone marrow and spleen cell transplantation.
3. The rebamipide-treated group (n = 6) included B6D2F1 mice 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. The volume of ophthalmic application was also selected based on the previously published studies that use 2-μL drop administration per mouse eye.

**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. 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.

**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. 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. Fluorescein agglutinin lectin binds to the N-acetylgalcosamine and N-acetylmuraminic acid residues present on the ocular surface glycocalyx and has been used in multiple studies, including human patients to stain corneal glycocalyx. 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 CO2 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.
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 lL 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 lL collected from each eye was pooled and added to 8 lL 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 RNaseasy Mini kit (RNaseasy 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 ΔΔCt 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 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.

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.

GVHD-Associated Dry Eye and Ocular Surface Mucins
IOVS | November 2019 | Vol. 60 | No. 14 | 4513

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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 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).

**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 the 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).
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 \(33 \pm 6.6\%\) 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 transplantation. 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 \((1.6 \pm 0.7 \text{ ng/mg protein})\) as compared with the levels in the control corneal homogenates obtained from mice that did not receive any transplantation \((0.8 \pm 0.4 \text{ ng/mg protein})\) 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 \pm 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 \pm 0.14-fold change in Muc4 mRNA levels (Fig. 6B), a 2.44 \pm 0.7-fold change in Muc16 mRNA (Fig. 6C), and a 0.265 \pm 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...
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 $3 \pm 0.25$ compared with a score of $8 \pm 0.5$ for the untreated (no eye drops) GVHD mice (Fig. 7B). It is interesting to note 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 $5 \pm 0.5$ 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 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.
GVHD-Associated Dry Eye and Ocular Surface Mucins

References


