SUPPLEMENTARY METHODS

Adenovirus Constructs
The human CD59 cDNA was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and PCR amplified using a forward primer containing an XhoI site (underlined) (5’cccctcgagtggacacatcataatggg3’) and a reverse primer with an EcoRV site (underlined) (5’cccggatatgacgagtttggagaag3’). The PCR product was gel purified and, after XhoI/EcoRV digestion, cloned into XhoI/EcoRV digested pShCAG (constructed by cloning a Sall/BamHI fragment of pCAGEN1 into XhoI/BgII digested pShuttle2) generating pShCAGCD59. Automated sequencing confirmed the full and correct CD59 sequence in the generated plasmid. This shuttle plasmid was then used to produce recombinant adenovirus as previously described1,2. AdEMPTY was generated similarly by recombining the PmeI linearized pShCAG with pAdEasy-1. The AdCAGGFP has been previously described1.

Western Blot Analysis
Cells were lysed in 50 mM Tris-HCl, pH 8.0/150 mM NaCl/0.1% Sodium dodecyl sulfate/1% Triton X-100 containing 2% (v:v) protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Media from the same cells were collected, centrifuged, passed through a 0.22 µm filter to remove any remaining cell debris and concentrated 10X using a Biomax centrifugal filter with a 10 KD pore size (Millipore Corporation, Billerica, MA). Lysates and concentrated media were run under non-reducing conditions on a 15% Tris-glycine SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA) and proteins transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Following blocking in 5% (w:v) skim milk (Becton Dickinson, Sparks, MD), the membrane was probed for human CD59 using a mouse anti-human CD59 monoclonal antibody (1:1,000 dilution; Clone Mem-43; Abcam, Cambridge, MA), followed by a horseradish preoxidase-conjugated goat anti-mouse antibody (1:10,000 dilution; Jackson Immunoresearch, West Grove, PA). Following stripping, and blocking as above, the same membrane was probed for β-Actin with a mouse anti-β-actin monoclonal antibody.
(1:5,000 dilution; Clone AC-15; Sigma-Aldrich, St. Louis, MO). Secondary detection was performed as described above.

**Trypan Blue Exclusion Assay**
Cells were treated as for the MAC deposition assay in cell culture except that immediately after washing away the serum, cells were incubated in 0.1% trypan blue solution (Invitrogen Corp., Carlsbad, CA) for 5 minutes. Subsequently, cells were washed twice with 1xPBS and fixed as described above.

**Subretinal Injections**
C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME), bred and maintained in a 12-hour light-dark cycle and cared for in accordance with federal, state and local regulations. Mice were anesthetized by intraperitoneal injection of xylazine (10 mg/ml)/ketamine (1 mg/ml). Subretinal injections were performed as previously described\(^6\) using the transcleral-transchoroidal approach with a 32-gauge needle attached to a 5 µl glass syringe (Hamilton, Reno, NV). One microliter of a control mixture containing nine parts AdEMPTY and one part AdCAGGFP (total of 3x10^8 virus particles) or a mixture containing nine parts AdCAGCD59 and one part AdCAGGFP (total of 3x10^8 virus particles) were injected into C57Bl/6J mice.

**Immunocytochemistry/Immunohistochemistry**
Fixed cells or tissues were incubated with primary mouse monoclonal antibodies to human CD59 (clone M-43) or human C5b-9 (clone aE11) (both 1:50 dilution, Abcam, Cambridge, MA) in 1xPBS containing 6% (w:v) normal goat serum (Jackson Immunoresearch, West Grove, PA) for 2.5 hours with gentle rotatory shaking. Secondary detection was performed similarly using a Cy3-conjugated goat anti-mouse antibody (1:400 dilution; Jackson Immunoresearch, West Grove, PA) for 1.5 hours in a dark chamber. For RPE65 immunostaining, primary RPE cells were pre-blocked and permeabilized in 1xPBS containing 6% (w:v) normal goat serum (Jackson Immunoresearch, West Grove, PA) and 0.25% (v:v) Triton X-100 (Fischer Bio-reagents,
Fair Lawn, NJ) for 1 hour. A mouse anti-RPE65 antibody (Novus Biologicals, Littleton, CO) was applied and primary and secondary detection were performed as above except that the antibody and washing solutions contained 0.25% (v:v) Triton X-100 (Fischer Bio-reagents, Fair Lawn, NJ). For mouse emmprin staining, goat anti-mouse emmprin antibody treated and fixed cells and tissues were blocked in PBS containing 6% (w:v) normal donkey serum (Jackson Immunoresearch, West Grove, PA) for 1 hour and secondary detection was performed using a Cy3-conjugated donkey anti goat antibody (1:400 dilution; Jackson Immunoresearch, West Grove, PA) in PBS containing 6% (w:v) normal donkey serum for 1.5 hours.

Image Analysis
Following immunocytochemistry, eyecups were flat mounted on slides and coverslipped. Corneas were sequentially dehydrated in 15% and 30% sucrose solution overnight, embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA), and 14 µm frozen sections were collected on glass slides. Cells and corneal sections were stained with 0.1 ug/ml 4',6-diamidino-2-phenylindole (DAPI), washed with 1xPBS and coverslipped. Images of experimental and control specimens were captured using identical settings with either an Olympus BX51 or an Olympus IX51 microscope equipped with Retiga EXi cameras and processed identically with Photoshop 9.0.2. For semi-quantitative analysis of MAC immunofluorescence or GFP expression original grayscale images were analyzed with ImageJ 1.38x. The area of interest was selected with either the freehand or polygon selection tool and mean fluorescence intensity per pixel was measured. Background fluorescence from similar images of untreated eyecups and corneas was subtracted from all measurements.
SUPPLEMENTARY RESULTS

Protection from MAC Deposition on Murine Corneal Endothelium by Adenovirus-Mediated Delivery of hCD59

Pre-treatment of murine corneas with the anti-mouse emmprin antibody followed by addition of NHS (final concentration 50% for 20 minutes at 37°C) resulted in extensive, bright MAC immunostaining on the corneal endothelium (Supplementary Fig. 5A). Minimal staining was observed on the endothelium of HI-NHS treated corneas.

To assess the efficacy of hCD59 to protect the corneal endothelium from human MAC deposition, we infected corneas ex vivo with the AdCAGCD59 or the control (AdCAGGFP) adenovirus. hCD59 expression on the corneal endothelium following ex vivo infection with the AdCAGCD59 was confirmed by immunohistochemistry using the anti-hCD59 antibody, while no staining for hCD59 was observed on control (AdCAGGFP)-infected corneas (Supplementary Fig. 5B).

hCD59 delivery to the corneal endothelium significantly protected those cells from human MAC deposition reducing MAC immunofluorescence intensity by 86% (p < 0.0001, Supplementary Fig. 5C) compared to delivery of GFP, which failed to protect the corneal endothelium resulting in MAC deposition levels, which were similar to those on the corneal endothelium of uninfected corneas. Moreover, the GFP expression on the corneal endothelium of NHS treated corneas appeared fragmented suggesting loss of endothelial cells due to damage by deposition of the MAC. This fragmentation was not observed on AdCAGGFP-infected corneas that were not exposed to NHS (Supplementary Fig. 5B and D).

The protection from MAC deposition on the corneal endothelium of AdCAGCD59-infected corneas could not have been due to a difference in emmprin expression and / or anti-emmprin antibody binding, as immunohistochemistry revealed no differences in emmprin immunostaining on the corneal endothelium between AdCAGCD59 and AdCAGGFP-infected, or uninfected corneas (Supplementary Fig. 5D).

In conclusion these data provide further confirmation of the potential of adenovirus delivered hCD59 to protect ocular tissues from MAC attack. Of note is the
fact that protection on the corneal endothelium was higher than that on the RPE. We hypothesize that this is due to the higher and more homogenous transduction of the endothelium of ex vivo-infected corneas and stress the utility of the cornea in the testing of the efficiency of complement regulators.

SUPPLEMENTARY REFERENCES