

GENETICS

RNA delivery to the corneal endothelium using charge-altering releasable transporters

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RNA therapies hold tremendous promise for treating genetic eye diseases. However, their development is limited by the lack of non-viral delivery platforms that can target specific ocular cell types. Here, we describe a charge-altering releasable transporter (CART) that delivers RNA selectively to the corneal endothelium, a non-regenerative cell layer whose dysfunction underlies several blinding conditions. We characterize the safety of CART-RNA nanoparticles in mice and show that they facilitate delivery of diverse RNA cargoes to the corneal endothelium, including circular RNA and CRISPR/Cas9. We verify that these nanoparticles can be redosed and apply them to achieve corneal gene editing. We further demonstrate CART transfection of corneal endothelial cells from a human donor in vitro and in a non-human primate in vivo, supporting the feasibility of clinical translation. Our findings establish CARTs as a platform for non-viral gene delivery to the eye, with the potential to treat corneal dystrophies and other vision disorders.

INTRODUCTION

RNA medicines have emerged as a promising therapeutic class to address a broad spectrum of pathologies, ranging from rare genetic disorders to widespread infectious diseases (1, 2). Paralleling the rise of RNA therapies have been advances in non-viral nucleic acid delivery platforms, such as lipid nanoparticles (LNPs) and polymeric nanocarriers (3–8). In contrast to viral vectors, these nanoparticle-based systems mediate transient gene expression, offering control over the timing of gene activity while minimizing the risk of genomic integration or immune activation (9–11). Together, these technologies made possible the development of mRNA vaccines against coronavirus disease 2019 (COVID-19) (12, 13) and are now being adapted for protein replacement therapies (14, 15), gene editing approaches (16, 17), and personalized cancer treatments (18, 19).

The eye is an attractive target for RNA medicines due to its accessible anatomy, small size, compartmentalized structure, and relative immune tolerance (20, 21). Indeed, pegaptanib (Macugen), an RNA aptamer injected into the vitreous of the eye to treat neovascular age-related macular degeneration (22), was among the first RNA-based drugs approved by the U.S. Food and Drug Administration (FDA) for any indication. Genetic eye diseases are particularly suitable candidates for RNA therapies given the potential to cure these conditions using gene editing tools like CRISPR/Cas9 (23). Such cargoes

are challenging to fit within the size constraints of adeno-associated viral (AAV) vectors, the most widely used vehicle for ocular gene delivery (24), but can be packaged as RNAs into nanoparticles to achieve in vivo editing (25, 26). Nanoparticle delivery of gene editing enzymes additionally avoids their long-term expression, limiting the off-target effects and immune reactions that can occur when these molecules are expressed from AAV vectors (27–29).

Previously, we described a class of biodegradable polymers called charge-altering releasable transporters (CARTs) that efficiently deliver RNA in vivo (6, 30–33). At acidic pH (<5.5), cationic CARTs electrostatically bind polyanionic RNA, forming stable nanoparticle complexes (Fig. 1A). Upon administration, these nanoparticles rapidly enter cells and self-degrade into neutral small molecules via a charge-altering mechanism, releasing the RNA into the cytosol. The tropism of CARTs can be tuned by modifying their chemical structures, allowing for targeted transfection of organs such as the spleen (30, 31), lungs (32, 34), and muscle (35, 36). More recently, CARTs have also been prepared as discrete entities termed DIGITs, which enable RNA delivery to red blood cells (37). Importantly, CARTs exhibited minimal toxicity in mice after intravenous or intramuscular delivery as measured by serum cytokine levels and histopathology (31, 35), suggesting that they may be safe to use in patients.

Here, we report the discovery of a CART that selectively transfects the corneal endothelium, a non-regenerative cell layer essential for maintaining corneal clarity. Genetic mutations in this cell type are responsible for several blinding diseases including Fuchs' endothelial dystrophy (38), the leading cause of corneal transplantation worldwide (39). We characterize the safety profile of CART-RNA nanoparticles in mouse eyes and show that they facilitate the delivery of diverse RNA cargoes to the corneal endothelium. We further demonstrate CART transfection of corneal endothelial cells from a human donor in vitro and in a non-human primate (NHP) in vivo, providing a proof of concept for translating this system to patients. Our findings establish CARTs as a platform for non-viral gene delivery to the eye, with the potential to treat corneal dystrophies and other vision disorders.

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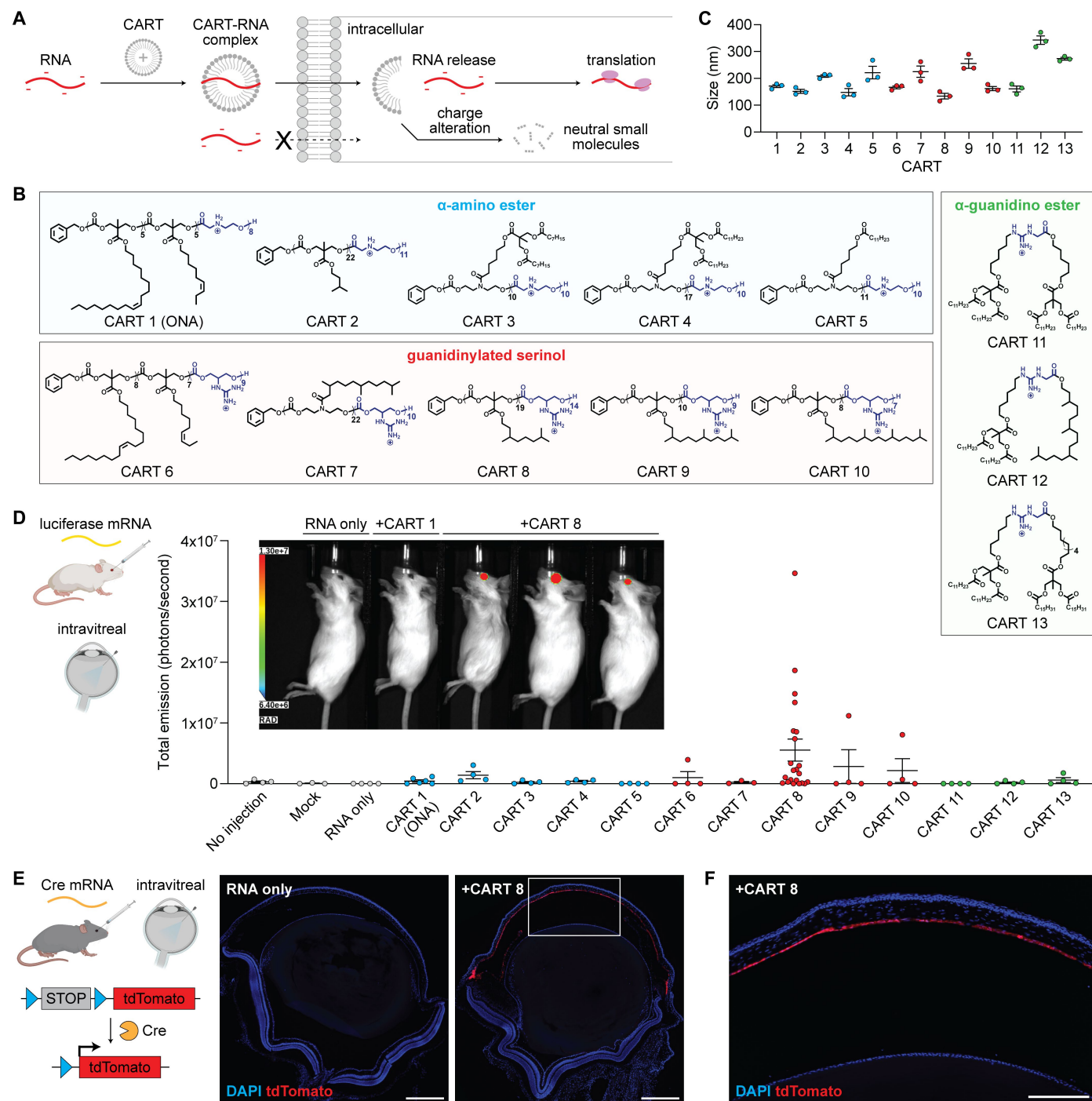


Fig. 1. Intravitreal screening identifies CARTs enabling ocular delivery of mRNA. (A) Proposed mechanism of CART delivery. Cationic CARTs electrostatically complex polyanionic RNA, protecting it during cell delivery. Once inside cells, CARTs undergo self-degradation into neutral small molecules, releasing the RNA into the cytosol. (B) Chemical structures of CARTs. Structures are grouped by their charge-altering motif (α -amino ester, guanidinylated serinol, or α -guanidino ester). (C) Particle size of CARTs complexed with 420 ng of luciferase mRNA at a 10:1 charge ratio in acidified 1x PBS (pH 5.5). Data are mean $\pm$ SEM. $n = 3$ technical replicates per group. (D) In vivo luminescence imaging of BALB/c mice without treatment or 12–16 hours after mock injection, intravitreal injection of luciferase mRNA only, or intravitreal injection of luciferase mRNA with the indicated CART. Eyes were dosed with 300 ng of RNA at a 10:1 charge ratio. Data are mean $\pm$ SEM. $n = 3-22$ eyes per group. (E) Whole eye sections from Ai9 mice 1 week after intravitreal injection of Cre mRNA only or Cre mRNA with CART 8. Eyes were dosed with 300 ng of RNA at a 10:1 charge ratio. $n = 4-6$ eyes per group. Scale bars, 500 μ m. (F) Anterior segment section from an Ai9 mouse 1 week after intravitreal injection of Cre mRNA with CART 8. Image location is indicated by white inset in (E). Image is representative of $n = 2$ of 6 eyes. Scale bar, 200 μ m. Created in BioRender. Wang, S. (2026) <https://BioRender.com/pg8axe3>.

RESULTS

Screening of CARTs for ocular delivery of mRNA

To determine if CARTs could transfect the eye, we synthesized a panel of 13 CARTs (Fig. 1B and fig. S1, A and B) spanning three distinct charge-altering motifs, each associated with a different mechanism of charge-cancellation and RNA release (fig. S2A) (6, 32, 37). This panel included ONA (CART 1), our most studied CART (30), as well as CARTs containing α -amino esters (CARTs 2–5) (31, 36), guanidinylnated serinols (CARTs 6–10) (32), or α -guanidino esters (CARTs 11–13) (37). It also encompassed a diverse selection of lipid moieties (unsaturated lipids for CART 1 and 6; isoprenoid lipids for CART 2, 7–10, and 12; fork lipids for CART 3, 4, and 11–13) and two types of polymeric backbones (e.g. CART 7 versus CART 9) to investigate the effects of hydrophobic substituents and lipid spacing (30, 31, 36). CARTs were formulated with mRNA encoding firefly luciferase by micropipette mixing, yielding average particle diameters of 134–343 nm (Fig. 1C). Polydispersity indices ranged from 0.1–0.3, indicating mostly uniform size distributions (fig. S2B), and all complexes carried a net positive surface charge (fig. S2C). For in vivo testing, we injected CART-RNA formulations into the vitreous cavity of the eye, a common route for ocular drug delivery.

We performed intravitreal injections of CARTs complexed with luciferase mRNA in albino mice and imaged their eyes after 12–16 hours. Formulations using α -amino or α -guanidino ester CARTs showed little to no change in luminescence compared to untreated, mock injected, or RNA only controls (Fig. 1D). In contrast, in a subset of eyes injected with guanidinylnated serinol CARTs, we observed strong luciferase activity, suggesting that this group of CARTs might facilitate ocular delivery of mRNA. We focused on CART 8 (BnO-MTCCit19-GSer14), which exhibited the highest luminescence, lowest surface charge, and most rapid RNA release kinetics among guanidinylnated serinol CARTs (fig. S2, D to H), and used Ai9 mice to assess its tropism in the eye. Following exposure to Cre recombinase, these animals constitutively express the tdTomato fluorescent protein (40), allowing identification of transfected cells. TdTomato was not detected in eyes that received Cre mRNA only but was robustly expressed in two of six eyes after intravitreal injection of Cre mRNA with CART 8 (Fig. 1E). Surprisingly, this expression was absent from the retina and instead localized to structures in the anterior segment, including the posterior surface of the cornea (Fig. 1F).

Transfection of the corneal endothelium by CART 8

We hypothesized that introducing CART 8 closer to the cornea would enhance the efficiency and consistency of corneal transfection. To test this, we first trialed topical delivery in Ai9 mice by placing a 5 μ L drop of CART 8 complexed with Cre mRNA directly onto the ocular surface. However, this route of administration failed to produce any tdTomato-positive cells in the cornea (fig. S3). We therefore explored intracameral delivery by injecting CARTs into the anterior chamber of the eye, granting them immediate access to the cornea and other anterior segment structures. Such injections are routinely performed in patients and can be done in a clinic setting using only topical anesthesia. As a control, we also formulated Cre mRNA into LNPs containing SM-102, the ionizable lipid used in the Moderna COVID-19 vaccine (41).

Intracameral delivery of Cre mRNA with CART 8 induced tdTomato expression in the corneas of Ai9 mice (Fig. 2A). In contrast, tdTomato was absent from eyes that received Cre mRNA only and was restricted to the iris and iridocorneal angle after injection of Cre mRNA with

CART 1 or SM-102 LNP (fig. S4A). Corneal expression of tdTomato was additionally observed in eyes that received CART 6 or CART 9, two of the other guanidinylnated serinol CARTs (fig. S4B). While CART 1, CART 6, and CART 10 showed partial transfection of the iris (figs. S4B and S5A), iris transfection with CART 8 was much more sparse (fig. S5B). Given tdTomato labeling of the posterior corneal surface (Fig. 2B), we reasoned that CART 8 transacted the corneal endothelium. Consistent with this, tdTomato expression in the cornea colocalized with immunostaining for Na⁺/K⁺-ATPase (Fig. 2C), a marker of corneal endothelial cells (42).

To evaluate the cell type-specificity of CART 8, we sorted tdTomato-positive cells from the anterior segments of Ai9 mice after intracameral injection of Cre mRNA with CART 8 (fig. S6A) and profiled their gene expression by RNA sequencing (RNA-seq). Compared to untreated anterior segments, tdTomato-positive cells were significantly enriched for corneal endothelial marker genes such as *Cdh2*, *Col4a3*, and *Slc4a4* (43, 44). Conversely, tdTomato-positive cells were significantly depleted for markers of the corneal epithelium (45), corneal stroma (43, 45, 46), conjunctiva and limbus (43, 45), and iris and ciliary body (47) (Fig. 2D and data S1). Together, these data demonstrate selective transfection of the corneal endothelium by CART 8 following intracameral administration.

Safety of intracameral CART 8 in mice

We previously reported that guanidinylnated serinol CARTs facilitate RNA delivery to the spleen and lungs (32). To determine if systemic transfection occurred following intracameral injection of CART 8, we harvested spleens and lungs from Ai9 mice. No tdTomato-positive cells were detected in these organs after administration of Cre mRNA with or without CART 8 (fig. S6, B and C), suggesting that the tropism of intracameral CART 8 was confined to the eye.

Our prior work also showed that adjusting the cation-to-anion charge ratio of CART-mRNA formulations can modify their transfection efficiency (6, 31, 32). We thus tested intracameral injections of CART 8 with Cre mRNA at different cation (guanidinium)-to-anion (phosphate) ratios using a fixed dose of Cre mRNA. Corneal flat mounts from Ai9 mice exhibited robust transfection of the endothelium at as low as a 1.25:1 charge ratio (fig. S7, A and B), with reduced transfection observed at much lower or higher ratios. TdTomato expression at this charge ratio was sparse in the iris and absent from the ciliary body (fig. S7C), indicating that CART 8 was still highly selective for the corneal endothelium.

Using a 1.25:1 charge ratio, we characterized the safety of intracameral CART 8 in mice by assessing its effect on corneal morphology and gene expression. At a gross level, eyes that received Cre mRNA with CART 8 displayed no obvious changes in appearance such as corneal edema, buphthalmos, or cataract formation (Fig. 3A). To measure the health of corneal endothelial cells, we performed immunostaining for zonula occludens-1 (ZO-1) to delineate cell junctions (48) (Fig. 3, B and C) and quantified endothelial cell density and size variation in the central cornea (fig. S8). We found no significant difference in endothelial cell density at one week or four months following injection of Cre mRNA with CART 8 relative to Cre mRNA alone (Fig. 3D and fig. S9, A and B). While there was a trend toward more variable cell size in eyes that received Cre mRNA with CART 8, this effect did not reach statistical significance at either time point (Fig. 3E and fig. S9B). In contrast, examination of corneas one week after injection of CART 8 without any RNA cargo revealed increased variability in endothelial cell size (fig. S9, D and

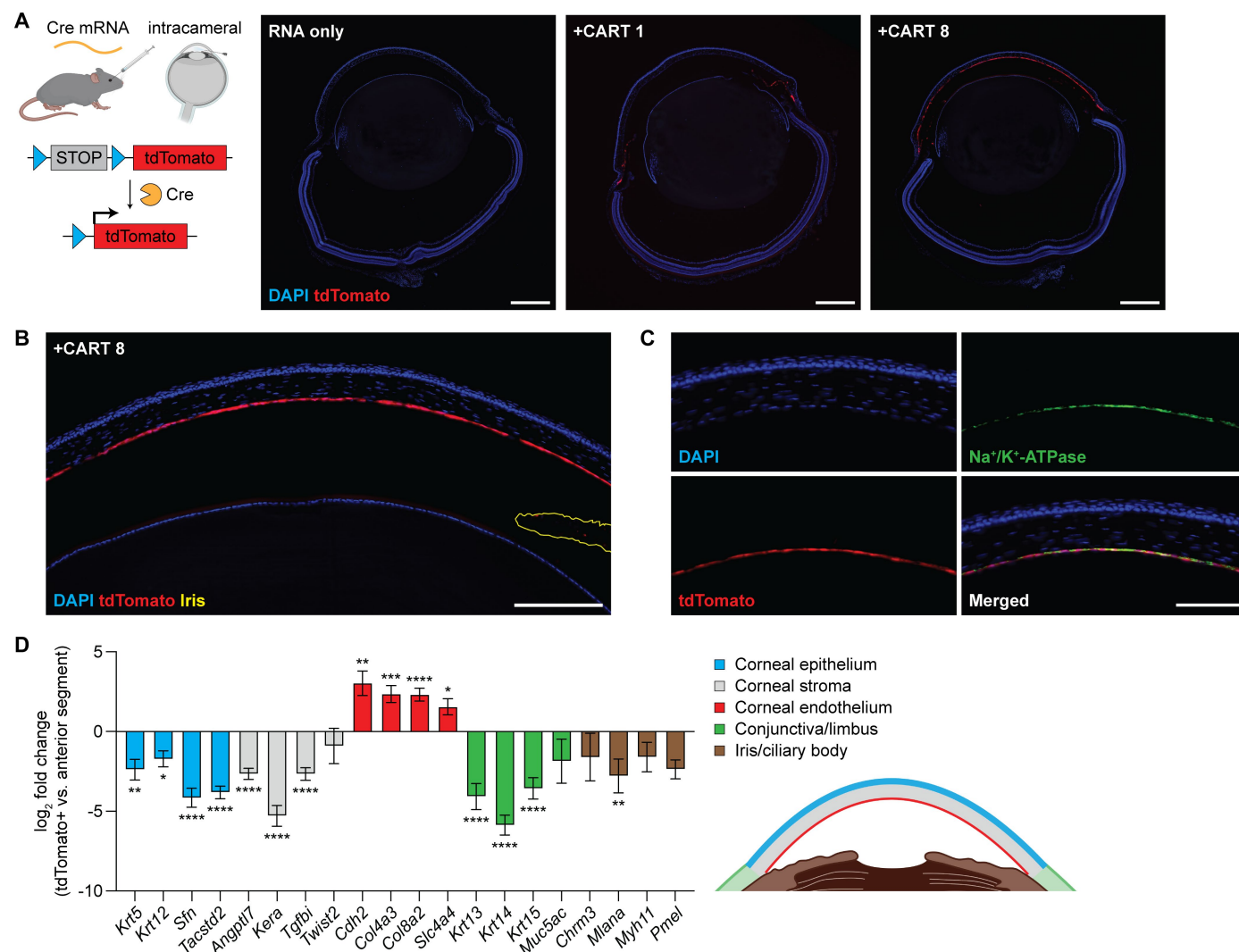


Fig. 2. Intracameral CART 8 selectively transfects the corneal endothelium. (A) Whole eye sections from Ai9 mice 1 week after intracameral injection of Cre mRNA only, Cre mRNA with CART 1, or Cre mRNA with CART 8. Eyes were dosed with 300 ng of RNA at a 10:1 charge ratio. $n = 4$ eyes per group. Scale bars, 500 μm . (B) Anterior segment section from an Ai9 mouse 1 week after intracameral injection of Cre mRNA with CART 8. Iris structures are outlined in yellow. $n = 4$ eyes. Scale bar, 200 μm . (C) Immunostaining for Na⁺/K⁺-ATPase in a corneal section from an Ai9 mouse 1 week after intracameral injection of Cre mRNA with CART 8. $n = 2$ eyes. Scale bar, 100 μm . (D) Up- and down-regulation of cell type-specific genes in tdTomato-positive anterior segment cells from Ai9 mice 1 week after intracameral injection of Cre mRNA with CART 8. TdTomato-positive cells were compared to cells from untreated anterior segments by RNA-seq. Colors denote different anterior segment cell types. Data are mean $\pm$ SEM. $n = 3$ –6 eyes per group. * adjusted $P < 0.05$, ** adjusted $P < 0.01$, *** adjusted $P < 0.001$, **** adjusted $P < 0.0001$. Created in BioRender. Wang, S. (2026) <https://BioRender.com/pg8axe3>.

E), possibly due to excess positive charge in the absence of RNA (fig. S9F). CART 8 may therefore trigger corneal endothelial toxicity when not complexed with polyanions.

We then conducted RNA-seq of mouse anterior segments 48 hours after intracameral injection of mRNA encoding enhanced green fluorescent protein (EGFP) with or without CART 8. As expected, EGFP mRNA was strongly up-regulated in eyes that received CART 8 (Fig. 3F and data S2), corroborating our sequencing results. Compared to injection of EGFP mRNA alone, addition of CART 8 significantly (absolute log₂ fold change ≥ 1 , adjusted $P < 0.05$) altered the expression of only nine endogenous genes, most of which possess immune functions (49–53). Importantly, intracameral CART 8 did not affect the expression of corneal endothelial genes (*Cdh2*,

Col4a3, *Col8a2*, *Slc4a4*) (43, 44), canonical pro-inflammatory cytokines (*Il1a*, *Il1b*, *Il6*, *Tnf*) (54), or markers of cell death (*Bax*, *Bcl2*, *Casp3*, *Fas*) (55). Supporting this, measurement of intraocular cytokines following delivery of EGFP mRNA with CART 8 compared to EGFP mRNA alone detected up-regulation of several chemokines, but no increases in IL-1 α , IL-1 β , IL-6 or TNF- α (fig. S10A). Likewise, we found no significant difference in corneal terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining 48 hours after injection of luciferase mRNA with or without CART 8 (fig. S9, G and H).

To evaluate the persistence of gene expression changes following CART 8 administration, we assayed three of the up-regulated genes in anterior segments four months after intracameral delivery of Cre

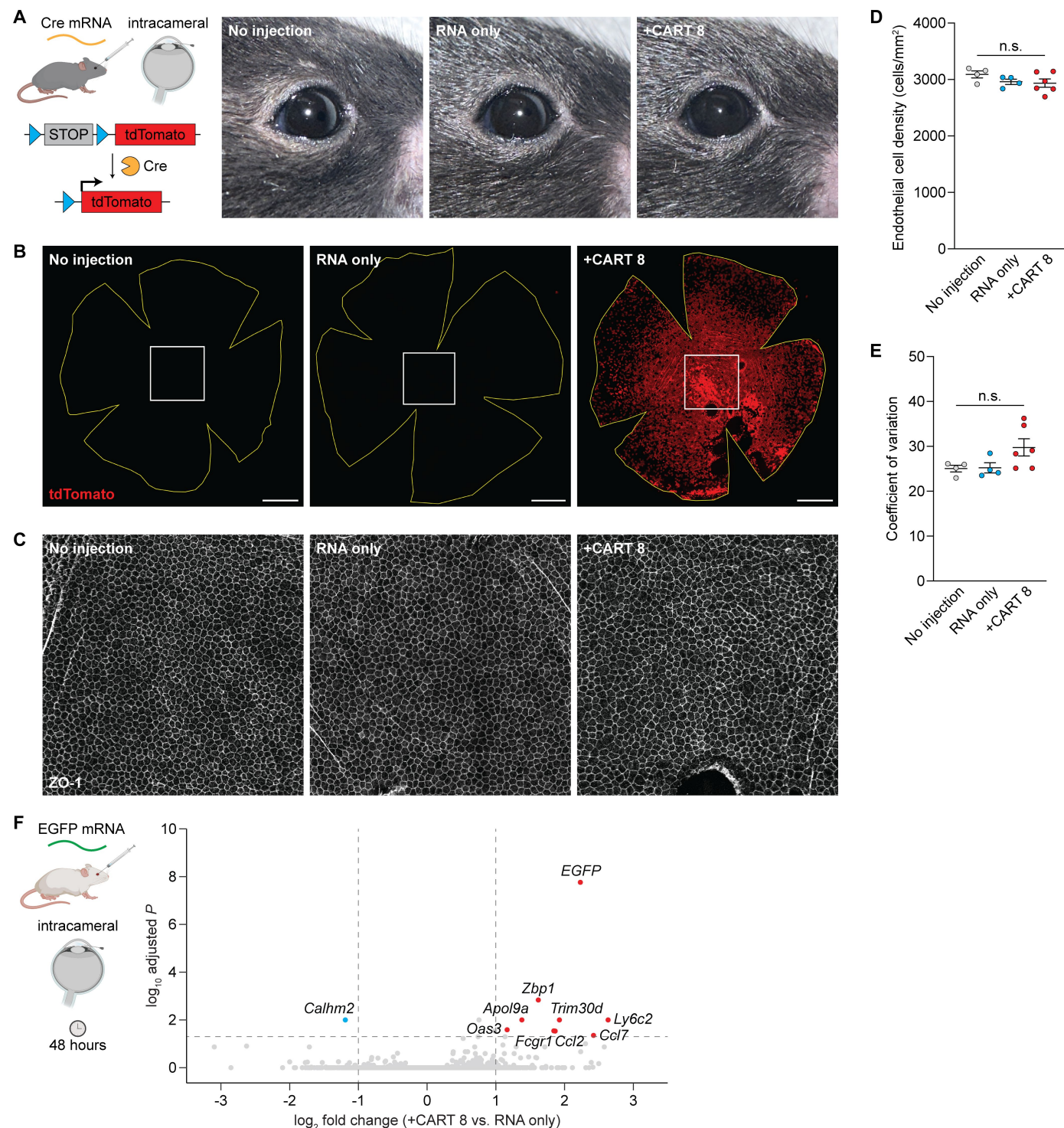


Fig. 3. Effect of intracameral CART 8 on corneal morphology and gene expression. (A) External photos of Ai9 mice without treatment or 1 week after intracameral injection of Cre mRNA only or Cre mRNA with CART 8. Eyes were dosed with 500 ng of RNA at a 1.25:1 charge ratio. $n = 4-6$ eyes per group. (B) Representative images of corneal flat mounts from Ai9 mice without treatment or 1 week after intracameral injection of Cre mRNA only or Cre mRNA with CART 8. $n = 4-6$ eyes per group. Scale bars, 500 μm . (C) Immunostaining for ZO-1 in corneal flat mounts from Ai9 mice without treatment or 1 week after intracameral injection of Cre mRNA only or Cre mRNA with CART 8. Image locations are indicated by white insets in (B). $n = 4-6$ eyes per group. (D and E) Quantification of corneal endothelial cell density (D) and coefficient of variation (E) in anti-ZO-1-stained corneal flat mounts. Data are mean $\pm$ SEM. $n = 4-6$ eyes per group. n.s., not significant. (F) Differentially expressed genes in anterior segments from C57BL/6 J mice 48 hours after intracameral injection of EGFP mRNA with CART 8 compared to EGFP mRNA only. Eyes were dosed with 500 ng of RNA at a 1.25:1 charge ratio. Red and blue dots denote significantly (absolute log₂ fold change ≥ 1 , adjusted $P < 0.05$) up- and down-regulated genes by RNA-seq, respectively. $n = 4-6$ eyes per group. Created in BioRender. Wang, S. (2026) <https://BioRender.com/pg8axe3>.

mRNA with or without CART 8. Quantitative polymerase chain reaction (qPCR) at this time point showed similar levels of *Ly6c2*, *Trim30d*, and *Zbp1* in both conditions (fig. S9C), suggesting that induction of these genes was transient.

CART 8 delivery of circular RNA and CRISPR/Cas9

Circular RNAs (circRNAs) represent an emerging class of RNA therapeutics exhibiting enhanced stability and durable expression (33). To determine if CART 8 could deliver circRNA to the corneal endothelium, we synthesized a circRNA encoding Cre recombinase. Cre circRNA resisted digestion by the exonuclease RNase R (fig. S11), validating its circular structure. Following intracameral injection in Ai9 mice, we detected tdTomato expression in the corneal endothelium of eyes that received Cre circRNA with CART 8, but not Cre circRNA only (Fig. 4A). These data indicate that CART 8 can deliver both mRNA and circRNA to corneal endothelial cells.

One advantage of RNA delivery systems is the potential for repeated administration. To confirm the feasibility of redosing CART 8, we performed serial intracameral injections of luciferase mRNA in albino mice. Consistent with our intravitreal experiments, eyes that received luciferase mRNA with CART 8 showed robust luciferase activity after 16 hours, while eyes that received luciferase mRNA only showed no change in luminescence (Fig. 4, B and C). Following resolution of luciferase activity by two weeks post-injection, we repeated intracameral delivery of luciferase mRNA with or without CART 8. At 16 hours after redosing, we observed luciferase activity comparable to initial levels, demonstrating that intracameral CART 8 could be administered more than once. Concentrations of intraocular cytokines following redosing were also highly similar to those measured after the first injection (fig. S10B), supporting the safety of repeated delivery.

We further tested if CART 8 was able to mediate corneal gene editing by complexing CART 8 with mRNA encoding the Cas9 nuclease and a single guide RNA (sgRNA) targeting the STOP cassette in Ai9 or Ai14 mice (Ai9 sgRNA) (56). Successful CRISPR/Cas9 editing deletes this STOP cassette, enabling tdTomato expression. As a control, we injected CART 8 and Cas9 mRNA with a sgRNA targeting Gal4, a gene not found in mice (56). We employed equal masses of Cas9 mRNA and sgRNA in all formulations as was previously reported for CRISPR/Cas9 LNPs (25, 57).

Initial attempts at CART 8 delivery of CRISPR/Cas9 using a 1.25:1 charge ratio led to only modest editing (fig. S12, A and B), potentially due to larger and more varied particle sizes (fig. S13) that prompted us to trial a higher charge ratio. Following intracameral injection of CART 8 complexed with Cas9 mRNA and Ai9 sgRNA at a 5:1 charge ratio, 1.3–4.9% of the cornea by area was tdTomato-positive, indicating successful gene editing (Fig. 4, D and E). In contrast, negligible tdTomato expression was seen after injection of Cas9 mRNA and the control sgRNA with CART 8. To increase editing, we administered a second dose of Cas9 mRNA and Ai9 sgRNA with CART 8 one week after the initial injection, resulting in tdTomato expression in 2.3–11.9% of the cornea. Sections through these eyes revealed confinement of tdTomato labeling to the posterior corneal surface (Fig. 4F), verifying that editing took place in corneal endothelial cells. Analysis of tdTomato expression at one month after dosing additionally confirmed the persistence of edited cells (fig. S12, C and D).

We wondered if CART 8 could be applied to edit a disease-associated gene in the cornea. Previously, Uehara *et al.* reported that CRISPR/Cas9-mediated disruption of *Col8a2* in corneal endothelial

cells rescues mice with early-onset Fuchs' endothelial dystrophy (58). As a proof of concept, we performed two intracameral injections of CART 8 complexed with Cas9 mRNA and a sgRNA targeting the *Col8a2* start codon in wild-type mice and measured editing at this locus using amplicon sequencing. *Col8a2* editing was detected in 4.0–18.8% of alleles in posterior corneas that received the *Col8a2* sgRNA, compared to only 0.1% of alleles in those that received the control sgRNA (Fig. 4G and fig. S12E). Collectively, these findings highlight the ability of CART 8 to deliver diverse RNA cargoes to the corneal endothelium, including CRISPR/Cas9 for gene editing.

CART 8 activity in translational models

We lastly explored if the functionality of CART 8 might translate to humans by evaluating its activity in primary human corneal endothelial cells (HCECs). These cells were isolated from a donor cornea and minimally passaged to best approximate their *in vivo* physiology (Fig. 5A). Following administration of EGFP mRNA with CART 8, we detected EGFP expression in HCECs (Fig. 5B and fig. S14, A to D), demonstrating transfection of these cells. Conversely, no expression was seen in untreated HCECs or those that received EGFP mRNA alone. HCECs showed no signs of reduced viability at 16 hours after treatment with CART 8-RNA complexes (fig. S14E). These results suggest that CART 8 may enable RNA delivery to the corneal endothelium not only in mice, but also in humans.

Using HCECs, we investigated the effect of small molecule inhibitors of clathrin-mediated endocytosis (chlorpromazine) (59), caveolae-mediated endocytosis (methyl- β -cyclodextrin) (60), macropinocytosis (EIPA) (61), cell-surface glycosaminoglycans (sodium chlorate) (62), and endosomal acidification (bafilomycin A) (63) on CART 8 transfection *in vitro*. Of these inhibitors, methyl- β -cyclodextrin produced the largest reduction in transfection efficiency (fig. S15A), implicating caveolae-mediated endocytosis as a major contributor to RNA delivery by CART 8. To determine if CART 8 administration might elicit neutralizing antibodies, we also measured HCEC transfection in the presence of plasma from treated or untreated mice. Plasma from mice that received intracameral injections of EGFP mRNA with CART 8 did not significantly affect transfection relative to plasma from control animals (fig. S15B), arguing against the formation of neutralizing antibodies.

We then assessed the tropism of CART 8 in an NHP model, the African green monkey. One eye was intracamerally injected with CART 8 complexed with 20 μ g of EGFP mRNA in a 50 μ L volume, while the other was injected with EGFP mRNA only. After 48 hours, eyes were collected for histology including hematoxylin and eosin (H&E) staining (Fig. 5C) and immunostaining for EGFP. Initial examination revealed EGFP signal near the limbus and posterior wall of both eyes even in the absence of primary antibody (fig. S16, A and B), consistent with autofluorescence from extraocular tissues. In the eye that received CART 8, we observed EGFP expression in corneal endothelial cells on the posterior corneal surface (Fig. 5, D and E). EGFP-positive cells were additionally found in the anterior iris, but not in the ciliary body, lens, or retina (Fig. 5, F and G). Although the overall structure of the eye was maintained following injection of CART 8, slight numbers of mixed polymorphonuclear and mononuclear cells were identified at the iridocorneal angle (fig. S16C), indicating mild anterior chamber inflammation. In summary, our findings confirm the ability of CART 8 to transfect the corneal endothelium in an NHP *in vivo*, supporting the clinical development of this delivery technology.

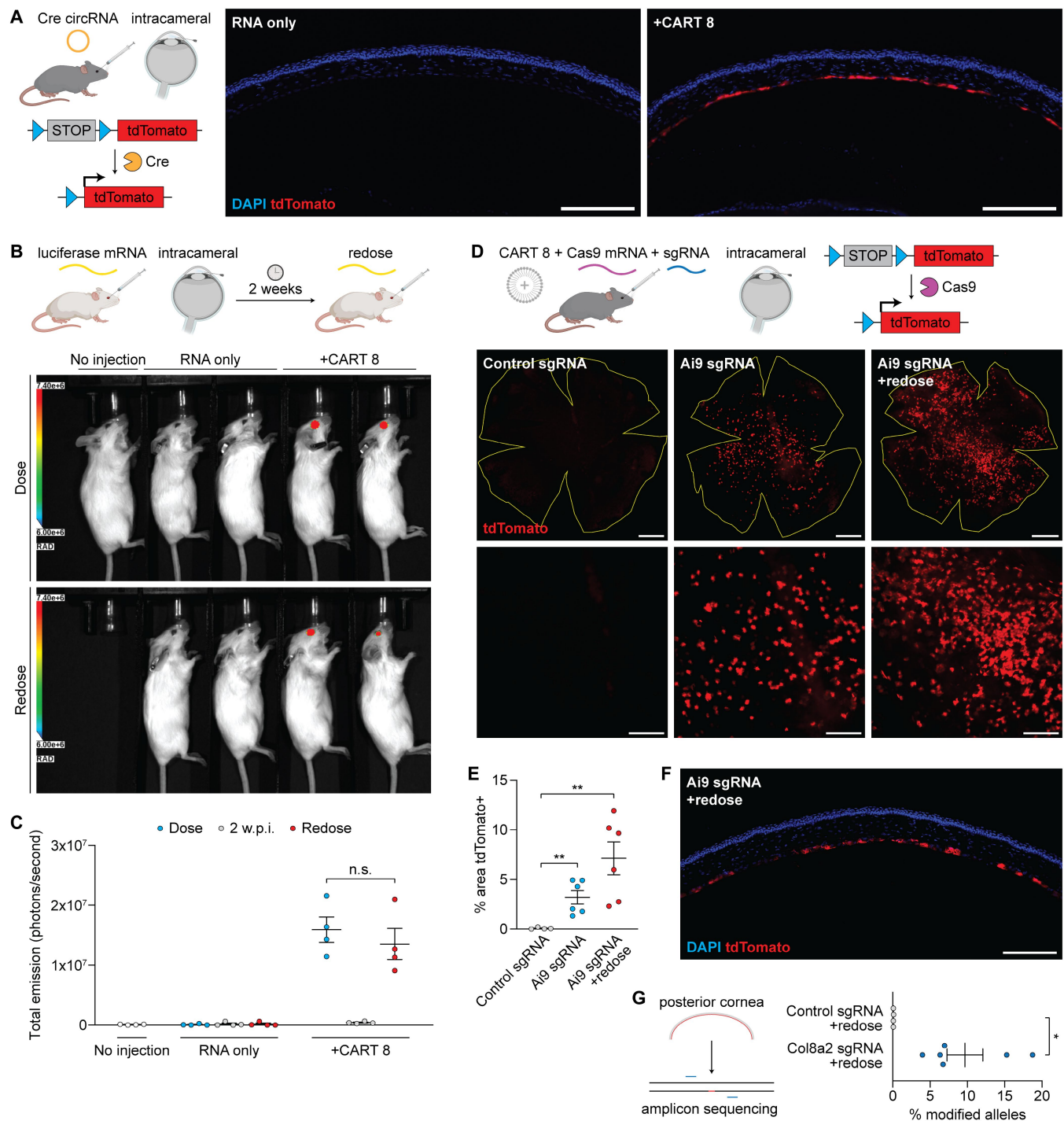


Fig. 4. CART 8 enables circular RNA expression and gene editing in the corneal endothelium. (A) Anterior segment sections from Ai9 mice 1 week after intracameral injection of Cre circRNA only or Cre circRNA with CART 8. Eyes were dosed with 500 ng of RNA at a 1.25:1 charge ratio. $n = 4$ eyes per group. Scale bars, 200 μm . (B) In vivo luminescence imaging of BALB/c mice without treatment or 16 hours after intracameral dosing or redosing of luciferase mRNA only or luciferase mRNA with CART 8. Eyes were dosed with 500 ng of RNA at a 1.25:1 charge ratio. Redosing was performed after 2 weeks. $n = 4$ eyes per group. (C) Quantification of in vivo luminescence imaging. Data are mean $\pm$ SEM. $n = 4$ eyes per group. n.s., not significant. (D) Representative images of corneal flat mounts from Ai9 mice 1 week after intracameral dosing or redosing of Cas9 mRNA and the indicated sgRNA with CART 8. Eyes were dosed with 600 ng of total RNA at a 5:1 charge ratio. Redosing was performed after 1 week. $n = 4$ –6 eyes per group. Scale bars, 500 μm (top), 100 μm (bottom). (E) Quantification of CRISPR/Cas9-mediated gene editing in Ai9 corneal flat mounts. Data are mean $\pm$ SEM. $n = 4$ –6 eyes per group. $**P < 0.01$ by two-tailed Student's t test. (F) Corneal section from an Ai9 mouse 1 week after intracameral redosing of Cas9 mRNA and Ai9 sgRNA with CART 8. $n = 2$ eyes. Scale bar, 200 μm . (G) Quantification of CRISPR/Cas9-mediated gene editing in C57BL/6 J mice at the *Col8a2* locus 1 week after intracameral redosing of Cas9 mRNA and the indicated sgRNA with CART 8. Data are mean $\pm$ SEM. $n = 4$ –6 eyes per group. $*P < 0.05$ by two-tailed Student's t test. Created in BioRender. Wang, S. (2026) <https://BioRender.com/pg8axe3>.

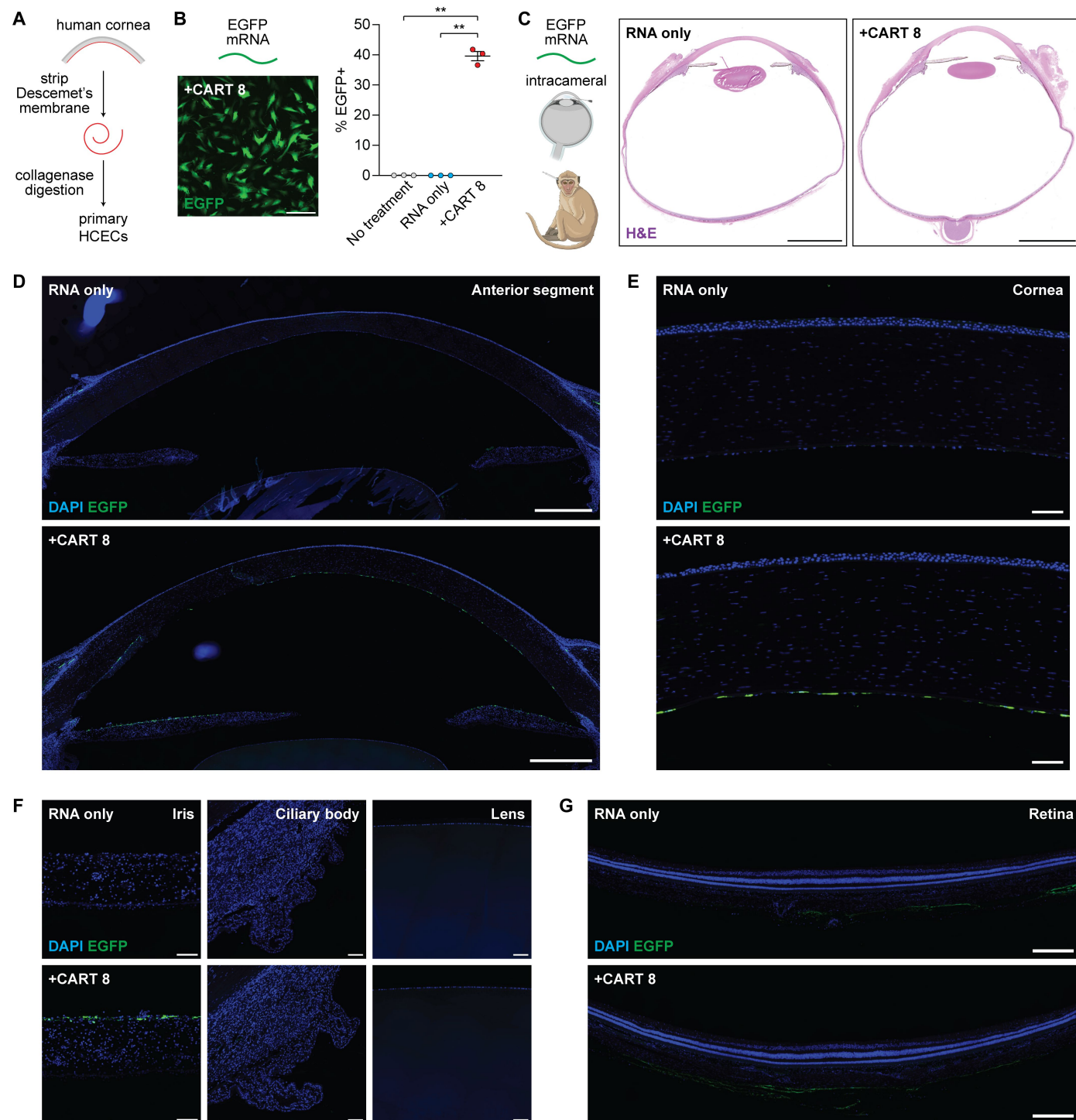


Fig. 5. CART 8 transfects corneal endothelial cells from a human donor and in a non-human primate. (A) Isolation of primary human corneal endothelial cells (HCECs). Descemet's membrane and the attached corneal endothelium were stripped from a donor cornea and digested with collagenase to liberate HCECs. Isolated cells were cultured for a maximum of three passages. (B) Left: Representative image of HCECs 16 hours after treatment with EGFP mRNA with CART 8. Scale bar, 200 μ m. Right: Flow cytometry quantification of EGFP expression in HCECs without treatment or 16 hours after treatment with EGFP mRNA only or EGFP mRNA with CART 8. Cells were treated with 100 ng of RNA per 96-well at a 4:1 charge ratio. Data are mean $\pm$ SEM. $n = 3$ biological replicates per group. $**P < 0.01$ by two-tailed Student's t test. (C) Hematoxylin and eosin (H&E) staining of whole eye sections from an NHP 48 hours after intracameral injection of EGFP mRNA only or EGFP mRNA with CART 8. Eyes were dosed with 20 μ g of RNA at a 3:2 charge ratio. $n = 1$ eye per group. Scale bars, 5 mm. (D to G) Immunostaining for EGFP in the indicated tissues of NHP eyes 48 hours after intracameral injection of EGFP mRNA only or EGFP mRNA with CART 8. $n = 1$ eye per group. Scale bars, 1 mm (D), 100 μ m (E and F), 500 μ m (G). Created in BioRender. Wang, S. (2026) <https://BioRender.com/pg8axe3>.

DISCUSSION

RNA therapies hold tremendous promise for treating genetic diseases. However, their application to vision disorders is still in its infancy, largely due to the lack of non-viral delivery platforms that can target specific ocular cell types. In this study, we identified a CART that delivers RNA selectively to the corneal endothelium, enabling therapeutic interventions such as gene augmentation and gene editing in these non-regenerative cells. CART 8 moreover transfected primary HCECs and the corneal endothelium in an NHP, underscoring its potential to deliver RNA to the same cell layer in patients. Our findings are especially relevant for sight-threatening diseases of the corneal endothelium, including Fuchs' endothelial dystrophy, posterior polymorphous corneal dystrophy, congenital hereditary endothelial dystrophy, and iridocorneal endothelial syndrome (64), opening new treatment avenues for these conditions outside of corneal transplantation. Endothelial cell transfection could additionally be useful during cataract surgery and other intraocular procedures to express genes that protect against endothelial cell loss (65) or to secrete factors that expedite postoperative healing (66).

Studies of LNPs in the eye have demonstrated RNA delivery to the retina, retinal pigment epithelium, and trabecular meshwork following intravitreal, subretinal, or intracameral administration (67–70). However, transfection of the corneal endothelium by LNPs remains inefficient (70), even when nanoparticles are injected directly into the corneal stroma (71). Although adenoviral vectors can successfully deliver genes to corneal endothelial cells (58, 72), their translation to patients faces multiple challenges, including pre-existing immunity against common serotypes and the potential to induce substantial inflammation (73, 74). While not as severe, ocular inflammation from LNPs has also been described, as evidenced by activation of retinal microglia and T cell infiltration in the choroid after subretinal delivery in an NHP (68). Here, we observed up-regulation of several chemokines and immune-related genes following CART 8 administration in mice and mild anterior chamber inflammation in an NHP, but no changes in corneal endothelial genes, canonical pro-inflammatory cytokines, or markers of cell death. In clinical applications, we anticipate that concurrent use of topical corticosteroids may further mitigate the safety risks of CART 8.

When delivered without a polyanionic counterpart, CART 8 led to greater variability in endothelial cell size, suggestive of toxicity to the corneal endothelium. This finding is not surprising as highly cationic molecules can destabilize cell membranes, triggering cell necrosis and inflammation (75, 76). Moreover, positively charged polymers and lipids such as polyethylenimine (PEI) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) have been shown to impair Na^+/K^+ -ATPase (76), which is abundantly expressed on corneal endothelial cells. In contrast, intracameral injection of CART 8-RNA complexes did not cause any significant morphological abnormalities. While there was a trend toward increased variation in endothelial cell size one week after injection of Cre mRNA with CART 8 compared to Cre mRNA only, this difference was less apparent at four months, supporting the long-term safety of CART 8 for RNA delivery.

Several limitations of our study should be acknowledged. Although we evaluated the effect of CART 8 on corneal endothelial cell density and morphology, we did not directly measure endothelial cell function. Parameters for gene editing including the sgRNA-to-Cas9 ratio and charge ratio were also not fully optimized, and we did not verify protein level changes after *Col8a2* editing or screen for

off-target CRISPR/Cas9 activity. Previously, Uehara *et al.* reported that an editing rate of 23.7% was sufficient to restore corneal endothelial function in the *Col8a2* mouse model of Fuchs' endothelial dystrophy (58). Follow-up experiments will be needed to determine if CART 8-mediated gene editing could similarly rescue corneal disease. We additionally demonstrated RNA delivery to the corneal endothelium in an NHP, but these results were limited to a single animal and not quantitatively assessed. Furthermore, unlike in mice, CART 8 transfected the anterior iris of the NHP. Given that iris tissue is highly vascular and rich in immune cells (77), this off-target expression could potentially introduce immune-related safety concerns. Finally, we did not examine the cumulative impact of repeated CART 8 administration on the corneal endothelium. Such data would be particularly relevant for diseased or injured corneas, where an already compromised endothelium might be less able to tolerate successive treatments. The efficiency of CART 8 transfection in these conditions may also differ from that in healthy eyes. It will thus be essential to test the therapeutic capabilities of CART 8 in an animal model of corneal endothelial dystrophy or damage prior to use in patients.

Why does CART 8 transfect the corneal endothelium? Although the exact reasons for endothelial cell selectivity remain to be determined, we hypothesize that the tropism of CART 8 is largely conferred by its guanidinylated serinols. In our initial screen, nearly every eye that exhibited luciferase activity had received a guanidinylated serinol CART, suggesting this charge-altering motif to be necessary for endothelial cell entry. Consistent with these observations, intracameral injection of CART 6 and CART 9, two other guanidinylated serinol CARTs, also resulted in endothelial cell transfection. However, guanidinylated serinols alone are not sufficient for endothelial cell targeting, as intracameral CART 10 did not transfect the corneal endothelium. One possibility is that the lower surface charge and faster RNA release kinetics of CART 8 relative to other guanidinylated serinol CARTs may have facilitated more efficient delivery of RNA. This delivery was strongly inhibited by methyl- β -cyclodextrin *in vitro*, supporting a dependence on caveolae-mediated endocytosis. The low frequency of luciferase expression with guanidinylated serinol CARTs in our initial screen likely reflected limited exposure to the cornea following intravitreal administration. Furthermore, negatively charged polymers in the vitreous such as hyaluronic acid might have trapped CART-RNA complexes (78, 79), preventing them from reaching posterior segment structures like the retina. Conversely, CART transfection after intracameral dosing may have benefited from natural convection currents in the anterior chamber, which circulate warmer fluid near the lens and iris toward the corneal endothelium (80). Future optimization of CARTs using these principles may enable RNA delivery to additional ocular cell types for gene therapy and gene editing applications.

MATERIALS AND METHODS

CART synthesis

CARTs were synthesized as previously described (6, 30–32, 36, 37). α -amino ester and guanidinylated serinol CARTs were synthesized as di- or tri-block polymers via anionic ring opening polymerization in Boc protected form and purified by dialysis against methanol. α -guanidino ester CARTs were synthesized in Boc protected form and purified via flash column chromatography (37). Boc protected CARTs were deprotected with 20% trifluoroacetic acid in dichloromethane

for 4 hours, dried, and dissolved in dimethyl sulfoxide (15–40 mM cationic concentration) before use.

CART-RNA formulations

CARTs were complexed with RNA in acidified 1x PBS (pH 5.5) containing 20% sucrose by rapid mixing using a micropipette for 20 seconds immediately prior to administration. Modified mRNAs encoding luciferase (L-7202), Cre (L-7211), EGFP (L-8101), and Cas9 (L-7206) were obtained from TriLink BioTechnologies. For size and zeta potential measurements, CART-RNA formulations were diluted with 1 mL of deionized water, immediately transferred to a disposable clear plastic cuvette, and measured using a NanoBrook Omni analyzer (Brookhaven Instruments). RNA doses and cation (ammonium or guanidinium)-to-anion (phosphate) charge ratios for all experiments are provided in figure legends.

RNA encapsulation and release assays

RNA encapsulation and release were measured using Quant-iT RiboGreen RNA Reagent (Thermo Fisher Scientific) in black 96-well plates. To quantify encapsulation, CART-RNA formulations were added to TE buffer (pH 5.5) containing 1:100 of RiboGreen fluorescent dye and fluorescence measured using a plate reader (480 nm excitation, 530 nm emission, 140 ms integration time). To quantify RNA release, CART-RNA formulations mixed with 10 μ L of 100 mM Na_2CO_3 were added to TE buffer (pH 7.5) containing 1:100 of RiboGreen fluorescent dye and fluorescence measured as above. Standard curves in TE buffers were used to calculate the percentage of RNA that was non-encapsulated or released.

Mouse injections

Mouse experiments were performed in 1–6-month-old BALB/c (#000651), Ai9 (#007909), C57BL/6 J (#000664), and Ai14 (#007914) mice obtained from The Jackson Laboratory. Both male and female mice were used and were randomly assigned to treatment groups. For intravitreal injections, animals were anesthetized using 2–3% inhaled isoflurane and pupils dilated with topical 1% tropicamide. A sharp 30-gauge needle was then inserted 1 mm posterior to the limbus to create a sclerotomy, through which a blunt 33-gauge Hamilton needle was introduced and 2 μ L of volume administered under direct visualization. Care was taken to ensure that the needle did not damage the lens or retina. For intracameral injections, mice were anesthetized as above. A microinjector pressure system (Picospritzer III) with a sharpened, pulled glass micropipette in a micromanipulator was used to inject reagents intracamerally through the cornea 1 mm anterior to the limbus, and 1 μ L of volume was administered under direct visualization without disturbing intraocular structures. Topical antibiotic ointment was applied to the eyes to aid in recovery. All mouse experiments were approved by the Institutional Animal Care and Use Committee at Stanford University (APLAC-14046).

In vivo imaging

In vivo luciferase activity was measured using an Ami HT optical imaging system (Spectral Instruments). Mice were anesthetized as above and injected intraperitoneally with 100 μ L of 30 mg/mL D-luciferin (Invitrogen). Mice were imaged after 5 minutes using default settings and an exposure time of 1 second. Luminescent activity was quantified using Aura 4.0 imaging software with equal-sized regions of interest centered on each eye.

External photos of mouse eyes were captured using an iPhone 14 Pro (Apple) with 3x magnification and flash on.

LNP formulation

SM-102 LNP was formulated by rapidly mixing SM-102 (Avanti), DSPC (Avanti), cholesterol (Avanti), and DMG-PEG 2000 (Avanti) at a 50:10:38.5:1.5 molar ratio with mRNA using a micropipette. RNA doses and charge ratios for experiments are provided in figure legends.

Mouse histology

Mouse eyes for cryosections were enucleated 1 week after injection. Following intravitreal injections, eyes were directly submerged in 4% paraformaldehyde (PFA) for 4 hours at room temperature. Fixed globes were subsequently washed with PBS, cryoprotected in a sucrose gradient, and embedded in a 1:1 mixture of 30% sucrose in PBS and optimal cutting temperature compound (Tissue-Tek) on dry ice. Following intracameral injections, eyes were first dissected in PBS to remove excess orbital tissue and a sclerotomy created before fixation in 4% PFA for 2 hours at room temperature. Fixed globes were then washed, cryoprotected, and embedded as above. Frozen globes were cut on a cryostat into 20- μ m sections, stained with 0.1 μ g/mL of 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen) to label nuclei, and mounted onto glass slides using ProLong Gold Antifade (Invitrogen). To detect Na^+/K^+ -ATPase, sections were additionally blocked with PBS containing 5% normal donkey serum (Jackson ImmunoResearch) and 0.1% Triton X-100 (Sigma-Aldrich) for 1 hour at room temperature, stained with 1:100 of rabbit anti- Na^+/K^+ + -ATPase antibody (Invitrogen, MA5-32184) in blocking buffer overnight at 4°C, and incubated with 1:1000 of Alexa Fluor 488 donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, 711-545-152) in PBS for 2 hours at room temperature.

Mouse eyes for corneal flat mounts were enucleated 1 week after injection unless otherwise specified. Using curved Vannas scissors, eyes were cut circumferentially at the limbus to isolate the cornea, which was fixed in 2% PFA for 30 minutes at room temperature. After PBS washes, corneas were relaxed with 4 radial incisions, carefully flattened onto glass slides with the endothelium facing up, and mounted using ProLong Gold Antifade. To detect ZO-1, corneas were additionally blocked with PBS containing 5% normal donkey serum and 0.1% Triton X-100 for 1 hour at room temperature, stained with 1:100 of rabbit anti-ZO-1 antibody (Invitrogen, 61-7300) in blocking buffer overnight at 4°C, and incubated with 1:1000 of Alexa Fluor 488 donkey anti-rabbit secondary antibody in PBS for 2 hours at room temperature.

Microscopy

Images of mouse cryosections and corneal flat mounts were acquired on either a Keyence BZ-X fluorescence microscope (2x, 10x, or 20x air objective) or Zeiss LSM 880 confocal microscope (10x air objective). All image analysis was performed using ImageJ. Anti-ZO-1-stained corneal endothelial cells were segmented and quantified using a custom ImageJ plugin in which the central 750 x 750 μ m region of each flat mount was subjected to automated background subtraction, thresholding, despeckling, watershedding, and particle counting. Endothelial cell density was calculated by dividing 10^6 by the mean cell area in μm^2 . Coefficient of variation was calculated by dividing the standard deviation of cell areas by their mean. Gene editing in Ai9 and Ai14 corneal flat mounts was quantified using a custom ImageJ plugin in

which each flat mount was subjected to automated background subtraction followed by thresholding to define tdTomato-positive cells. Editing was calculated as the percent area of each cornea that was tdTomato-positive. TUNEL staining in corneal flat mounts was quantified using a custom ImageJ plugin in which the central 750 x 750 μm region of each flat mount was subjected to automated background subtraction, thresholding, despeckling, and particle counting. Custom ImageJ plugins are available at <https://zenodo.org/records/19344868>.

Flow cytometry and cell sorting

Fluorescence-activated cell sorting (FACS) of tdTomato-positive cells was performed on a BD FACSARIA II. Eyes from Ai9 mice were cut circumferentially 1 mm posterior to the limbus to isolate the cornea, iris, and part of the ciliary body. To obtain single-cell suspensions, isolated tissues were digested with 625 units/mL of collagenase type I (Sigma-Aldrich) in PBS for 45 minutes at 37°C followed by 0.25% Trypsin-EDTA (Gibco) for 10 minutes at 37°C (81), then washed with PBS and stained with 0.1 $\mu\text{g}/\text{mL}$ of DAPI to exclude dead cells. TdTomato-positive cells were collected from eyes that received Cre mRNA with CART 8. Two eyes were pooled for each sample and a minimum of 500 cells sorted. Anterior segment control cells were collected from eyes without treatment. One eye was used for each sample and 1,000 cells sorted.

Flow cytometry of mouse spleen, mouse lung, and HCECs was performed on an Attune NxT flow cytometer. Isolated spleens were mashed into a cell suspension, filtered through a 70 μm cell strainer, treated with RBC Lysis Buffer (Tonbo) to lyse red blood cells, washed with PBS, and stained with 0.1 $\mu\text{g}/\text{mL}$ of DAPI. Isolated lungs were first digested with 8 units/mL of DNase I (New England Biolabs) and 125 units/mL of collagenase type I in PBS for 45 minutes at 37°C, then processed identically to spleens. HCECs were dissociated using 0.05% trypsin-EDTA (Gibco), washed with PBS, and stained with 0.1 $\mu\text{g}/\text{mL}$ of DAPI. All FACS and flow cytometry data were analyzed using FlowJo 10.

RNA isolation and sequencing

For RNA-seq of sorted cells, total RNA was collected using a Quick-RNA Microprep Kit (Zymo) and sent to Azenta Life Sciences (South Plainfield, NJ, USA) for ultra-low input RNA-seq library construction and sequencing with a target depth of 20 million reads per sample. The resulting FASTQ files were mapped to the mm10 genome using Bowtie2 (version 2.3.4.1), filtered for a MAPQ score > 10 using SAMtools (version 1.16.1), and subjected to PCR duplicate removal using Picard MarkDuplicates to generate BAM files (82–84). Gene expression counts were then obtained from BAM files using HTSeq (version 2.0.1) and differentially expressed genes identified using DESeq2 (version 1.26.0) (85, 86).

For RNA-seq and qPCR of anterior segments, eyes were cut circumferentially 1 mm posterior to the limbus to isolate the cornea, iris, and part of the ciliary body. Tissues were homogenized using a handheld pellet pestle (Kimble Chase) in 300 μL of RNA Lysis Buffer (Zymo) and centrifuged at 16,000 $\times g$ for 5 minutes. Total RNA was then collected from the supernatant using a Quick-RNA Microprep Kit (Zymo) following manufacturer's instructions. For RNA-seq, RNA was sent to Azenta Life Sciences for library construction and sequencing, mapped to a custom mm10 genome containing the EGFP sequence, and analyzed as described above. For qPCR, cDNA was synthesized using HiScript IV All-in-One Ultra RT SuperMix (Vazyme) and amplified using SupRealQ Ultra Hunter SYBR qPCR

Master Mix (Vazyme) on a CFX96 real-time PCR detection system (Bio-Rad). Reactions were performed in triplicate with expression normalized to the housekeeping gene *Gapdh*. Sequences for qPCR primers are listed in table S1.

TUNEL assay

TUNEL staining was performed using the Click-iT Plus TUNEL Assay for In Situ Apoptosis Detection (Invitrogen) following manufacturer's instructions. For DNase I treatment, corneas from untreated mice were fixed, permeabilized, and incubated with 1 unit of DNase I for 30 minutes at room temperature.

Cytokine measurements

Anterior segments were isolated as described above and placed in 100 μL per sample of RIPA lysis and extraction buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Following sonication and centrifugation to remove debris, the supernatant was collected and submitted to the Human Immune Monitoring Center at Stanford University for cytokine profiling. Cytokine levels were measured using mouse 48-plex Procarta kits (Thermo Fisher Scientific) following manufacturer's instructions. Each sample was measured in duplicate, and plates were read using a Luminex 200 or a FM3D FlexMap instrument with a lower bound of 50 beads per sample per cytokine.

Circular RNA synthesis

Cre circRNA was synthesized using methods previously described (33). Briefly, the Cre coding sequence was cloned into the pRC0940 plasmid backbone and PCR amplified to synthesize the circRNA template. In vitro transcription was performed using a HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) with 5% substitution of 5-methylcytidine triphosphate (TriLink BioTechnologies) for cytidine triphosphate. Column purified RNA was digested with 0.5 units of RNase R (Biosearch Technologies) per microgram of RNA for 30 minutes at 37°C to isolate circRNA.

Guide RNAs

Ai9 (5'-AAGTAAACCTCTACAAATG-3'), control (5'-AACGAC-TAGTTAGGCGTGTA-3'), and Col8a2 (5'-CCCATCCACAGACGC-CATGC-3') sgRNAs with standard 2'-O-methyl and 3' phosphorothioate modifications were obtained from Synthego and resuspended at a concentration of 1.6 $\mu\text{g}/\mu\text{L}$ in acidified 1x PBS (pH 5.5) containing 20% sucrose. A 1:1 mass ratio of Cas9 mRNA to sgRNA was used in all formulations.

Amplicon sequencing

The posterior cornea (corneal endothelium, Descemet's membrane, and posterior stroma) was mechanically separated from mouse corneas and processed using a Quick-DNA Microprep Kit (Zymo) following manufacturer's instructions to isolate genomic DNA. Nested PCR was performed using outer primers for 8 cycles followed by inner primers for 30 cycles to amplify the *Col8a2* locus (table S1). Amplicons were sent to Azenta Life Sciences for Amplicon-EZ sequencing with a target depth of 50,000 reads per sample. The resulting FASTQ files were analyzed using CRISPResso2 with default settings (87). Modified alleles were defined as those with any addition, deletion, or substitution within a 40-bp window centered 3 bp upstream of the protospacer adjacent motif (PAM) sequence.

Human corneal endothelial cell culture

Primary HCECs were isolated as previously described (88) from a 44-year-old male donor cornea procured by the San Diego Eye Bank under protocols approved by the Eye Bank Association of America. Briefly, Descemet's membrane and the attached corneal endothelium were stripped from the stroma and incubated in 0.2% collagenase type II (Worthington Biochemical) in PBS for 1 hour at 37°C. Cells were then pelleted and resuspended in endothelial cell growth medium 2 (EGM-2) with premixed supplements (PromoCell). Cells were grown on plates coated with FNC Coating Mix (Athena Enzyme Systems) at 37°C and 5% CO₂ for a maximum of three passages and sub-cultured using 0.05% trypsin-EDTA (Gibco).

In vitro transfection of HCECs was performed in a 96-well flat bottom plate at 40–60% confluency. For each well, culture medium was reduced to 25 µL of EGM-2 and 2 µL of EGFP mRNA with or without CART 8 introduced. After two hours, 100 µL of EGM-2 was added. CART 8 was complexed with EGFP mRNA in acidified 1x PBS (pH 5.5) containing 20% sucrose. Small molecule inhibitors (chlorpromazine, methyl-β-cyclodextrin, EIPA, sodium chlorate, and bafilomycin A) were obtained from Sigma-Aldrich and added to cell culture media 30 minutes before transfection, except for sodium chlorate which was added 48 hours prior. Cells were imaged on an EVOS M5000 microscope (Invitrogen) using a 10x air objective.

Neutralizing antibody assay

Blood was collected from the retro-orbital sac of mice using a 31-gauge insulin syringe and mixed with PBS containing 5 mM of EDTA to prevent clotting. The resulting mixture was centrifuged at 2,000 x g for 10 minutes and the plasma supernatant collected. Plasma from two mice was combined per condition and added to cell culture media 30 minutes before transfection.

NHP injections

Intracameral injections were performed in an adult male African green monkey (*Chlorocebus sabaeus*). The animal was anesthetized with intramuscular ketamine (8 mg/kg) and xylazine (1.6 mg/kg) and pupils dilated with topical 10% phenylephrine and 1% cyclopentolate. Topical 0.5% proparacaine was then administered, an eye speculum placed, and the ocular surface rinsed with 5% Betadine solution followed by sterile 0.9% saline. Intracameral injection was performed in each eye using a 31-gauge insulin syringe (UltiCare VetRx U-100) inserted through the temporal cornea 2 mm anterior to the limbus. A volume of 50 µL containing 20 µg of EGFP mRNA with or without CART 8 was administered under direct visualization without disturbing intraocular structures. Topical antibiotic ointment was applied to the eyes to aid in recovery. All NHP experiments were approved by the Institutional Animal Care and Use Committee of the St. Kitts Biomedical Research Foundation (AC24256–004).

NHP histology

The animal was euthanized and eyes enucleated 48 hours after injection. Following removal of excess orbital tissue, eyes were submerged in Davidson's fixative for 24 hours at room temperature, washed with PBS, and trimmed horizontally. Tissues were subsequently dehydrated through gradient alcohols, cleared in xylene, and infiltrated with paraffin wax for embedding. Paraffin-embedded tissue blocks were cut on a microtome into 4-µm sections and either stained with hematoxylin and eosin, or 1:1000 of rabbit monoclonal anti-GFP antibody (Abcam, ab183734) for 1 hour at ambient temperature. Binding

of the primary antibody was visualized with Discovery OmniMap anti-rabbit HRP (Roche, 760–4311) and a Cy5 fluorophore (Roche, 760–238) on a Ventana Discovery Ultra immunostainer (Roche). For some slides, primary anti-GFP antibody was omitted as a negative control. Whole slides were imaged on a Panoramic SCAN II digital slide scanner (3DHiTech) using 20x objective magnification.

Statistical analysis

Group data are shown as mean ± SEM. Two-tailed Student's *t* tests were used to compare experimental groups and were performed using GraphPad Prism. *P* < 0.05 was considered statistically significant.

Supplementary Materials

The PDF file includes:

Figs. S1 to S16

Table S1

Legends for data S1 and S2

Other Supplementary Material for this manuscript includes the following:

Data S1 and S2

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RNA delivery to the corneal endothelium using charge-altering releasable transporters

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Effectiveness of adult zoster vaccination in preventing a first episode of zoster keratitis: A large-scale database study

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ABSTRACT

Purpose: The adult recombinant zoster vaccine (Shingrix) has demonstrated effectiveness in preventing herpes zoster ophthalmicus (HZO); however, its effectiveness in preventing zoster keratitis has not been directly evaluated. Given the wide variability in reported keratitis rates among patients with HZO and the potentially sight-threatening nature of this complication, this study was conducted to address this knowledge gap.

Methods: This retrospective, multicenter cohort study was conducted using a global federated electronic health record network. All patients were aged ≥ 50 years and had no prior diagnosis of zoster ocular disease. The Shingrix-vaccinated cohort was compared with two control groups with no prior Shingrix exposure: patients with presbyopia and patients who received the quadrivalent influenza vaccine. Extensive 1:1 propensity score matching was performed. After matching, 571,746 patients were included in the presbyopia comparison and 626,697 in the influenza vaccine comparison. Primary and secondary outcomes were incident zoster keratitis and conjunctivitis, respectively, over a 5-year follow-up period. Cohort validity was evaluated using positive and negative controls.

Results: Shingrix-vaccinated patients demonstrated a significantly reduced risk of zoster keratitis (HR, 0.50; 95% CI [0.37, 0.67]; $p < 0.0001$) and zoster conjunctivitis (HR, 0.53; 95% CI [0.35, 0.81]; $p = 0.0025$) when compared with presbyopia controls. Similar results were observed in the comparison with influenza-vaccinated controls, with a reduced risk of zoster keratitis (HR, 0.56; 95% CI [0.39, 0.81]; $p = 0.0016$) and conjunctivitis (HR, 0.37; 95% CI [0.24, 0.57]; $p < 0.0001$).

Conclusions: This study supports the potential role of Shingrix in preventing herpes zoster keratitis.

1. Introduction

Herpes zoster is the clinical manifestation of varicella-zoster virus (VZV) reactivation and affects approximately 20% to 30% of individuals over the course of their lifetime. Herpes zoster ophthalmicus (HZO) is caused by reactivation of the virus in the ophthalmic branch (V1) of the trigeminal nerve, which accounts for 8% to 20% of all zoster cases [1,2]. Ocular involvement is common, occurring in up to 70% of cases, and may include nearly any ocular structure [1–3].

Herpes zoster keratitis is a common and potentially vision-threatening complication of HZO, occurring in approximately 13%–76% of cases [2]. VZV can affect the epithelium, stroma, and endothelium, with clinical manifestations ranging from acute dendritic keratitis to chronic stromal keratitis and neurotrophic keratopathy. These

conditions may result in corneal scarring and neovascularization, visual impairment, and, in severe cases, the need for corneal transplantation [2,4–6].

The recombinant zoster vaccine (Shingrix; VZV glycoprotein E) is highly effective in preventing a first episode of herpes zoster. In pivotal phase 3 randomized controlled trials, Shingrix demonstrated a vaccine efficacy of 97.2% in adults aged ≥ 50 years over a median follow-up of 3.2 years [7]. In addition, large real-world cohort studies have shown up to 89% effectiveness in preventing HZO [8,9]. It is approved for the prevention of herpes zoster in all adults aged 50 years and older, as well as in adults aged 18 years and older who are at increased risk due to immunodeficiency or immunosuppression. The vaccine is administered in two doses, with the second dose given 2 to 6 months after the first [10].

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Despite its well-established protective role, no studies to date have directly evaluated vaccine effectiveness specifically against VZV keratitis; instead, prior work has focused on HZO as a broader clinical entity. This gap is particularly important given the wide variability in reported keratitis rates among patients with HZO. Moreover, zoster keratitis is a clinically significant ocular complication that may substantially impair patients' quality of life, increase long-term ophthalmologic and corneal service utilization, and impose an economic burden on both patients and healthcare systems. To address this knowledge gap, we conducted a large, multicenter cohort study using a global electronic health record (EHR) network encompassing data from over 190 million patients to investigate whether Shingrix vaccination is associated with a reduced risk of first-episode VZV keratitis and to quantify the magnitude of this risk reduction.

2. Methods

2.1. Data source and study setting

We conducted a retrospective cohort study using TriNetX (<https://live.trinetx.com>), a large federated EHR network covering more than 190 million patients from over 160 healthcare organizations (HCOs) across North America, Europe, and Asia. The database provides standardized access to de-identified patient data, including demographics, diagnoses, procedures, medications, laboratory results, and healthcare utilization metrics. This analysis was conducted on December 12, 2025, following the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines. In accordance with TriNetX's privacy policy, the names and locations of participating HCOs are not disclosed. Because only de-identified data were used, the study was conducted in compliance with the Health Insurance Portability and Accountability Act (HIPAA) and was deemed exempt from institutional review board (IRB) approval.

2.2. Study population

The Shingrix vaccinated cohort included patients aged ≥ 50 years who received two doses of the VZV glycoprotein E vaccine (RxNorm 1986821), administered 2 to 6 months apart, in accordance with the recommended vaccination schedule [10]. Receipt of the second vaccine dose was defined as the index event. Patients with a prior diagnosis of zoster ocular disease (ICD-10-CM B02.3) recorded before any Shingrix vaccination were excluded. This code encompasses zoster ocular disease unspecified (ICD-10-CM B02.30), zoster conjunctivitis (ICD-10-CM B02.31), zoster iridocyclitis (ICD-10-CM B02.32), zoster keratitis (ICD-10-CM B02.33), zoster scleritis (ICD-10-CM B02.34), other herpes zoster eye disease (ICD-10-CM B02.39) and herpes zoster with ophthalmic complications (ICD-10-CM 053.2).

2.3. Control cohorts

Two control cohorts were created. The first consisted of patients aged ≥ 50 years with a diagnosis of presbyopia (ICD-10-CM H52.4) who had no prior diagnosis of zoster ocular disease (ICD-10-CM B02.3). This was defined as the index event. Patients with any documented receipt of the VZV glycoprotein E vaccine were excluded. Presbyopia was selected as a comparator diagnosis because it represents a common, age-related condition that is associated with ophthalmic care in adulthood while not being inherently associated with significant systemic comorbidity. The second consisted of patients aged ≥ 50 years who received the influenza quadrivalent vaccine (CPT 90685/90686/90687/90688) who had no prior diagnosis of zoster ocular disease (ICD-10-CM B02.3). This was defined as the index event. Patients with any documented receipt of the VZV glycoprotein E vaccine were excluded. This design was chosen to reduce healthy user bias and account for differences in healthcare-seeking behavior. Individuals who receive vaccinations, such as

Shingrix or influenza, are more likely to engage with the healthcare system and have more complete clinical documentation [11].

2.4. Propensity score matching (PSM)

Extensive PSM in a 1:1 ratio was performed using demographic, systemic, ocular, medication, and healthcare utilization variables assessed within the 3 years to 1 day before the index event. Demographics included age, sex, and race/ethnicity (White, Black or African American, Asian, Hispanic or Latino). Systemic clinical covariates included diabetes mellitus (E08-E13), hemoglobin A1c/hemoglobin total in Blood (TNX curated 9037), hypertensive diseases (I10-I1A), hyperlipidemia (E78.5), cholesterol in LDL [Mass/volume] in serum or plasma (TNX curated 9002), cholesterol in HDL [Mass/volume] in serum or plasma (TNX curated 9001), overweight and obesity (E66), ischemic heart diseases (I20-I25), chronic kidney disease (N18), malignant (primary) neoplasm (C80.1), malignant neoplasms of lymphoid, hematopoietic and related tissue (C81-C96), alcohol related disorders (F10), tobacco use (Z72.0), immunodeficiency (D84.9), human immunodeficiency virus [HIV] disease (B20), endocrine, nutritional and metabolic diseases (E00-E89), diseases of the musculoskeletal system and connective tissue (M00-M99) and mental, behavioral and neurodevelopmental disorders (F01-F99). Ocular clinical covariates included diseases of the eye and adnexa (H00-H59), other disorders of cornea (H18), dry eye syndrome (H04.12) and surgical procedures on the eye and ocular adnexa (CPT 1009727). Medication variables encompassed corticosteroids for systemic use (ATC H02), immunosuppressants (ATC L04) and oncology treatments (TNX curated TX). Healthcare utilization variables included ambulatory and emergency visits and ophthalmology services and procedures (CPT 1012793). Covariate imbalance was defined as a standardized mean difference > 0.1 with $P < 0.05$.

2.5. Outcomes

The primary outcome was incident zoster keratitis (ICD-10-CM B02.33), and the secondary outcome was incident zoster conjunctivitis (ICD-10-CM B02.31). All outcomes were defined a priori and assessed over a 5-year follow-up period, with a 30-day delay from the index event to the start of follow-up to allow adequate time for vaccine-induced immunity to develop [12]. This approach was chosen to minimize the risk of selective outcome reporting, which can occur in large database studies when outcomes are defined post hoc. Outcomes reflect incident cases recognized in routine clinical practice.

To evaluate the validity of the cohort design, we included both positive and negative control outcomes. Positive control variables consisted of: (1) VZV infection with central nervous system (CNS) involvement: zoster encephalitis (ICD-10-CM B02.0) or zoster meningitis (ICD-10-CM B02.1) or zoster with other nervous system involvement (ICD-10-CM B02.2); and (2) disseminated zoster infection (ICD-10-CM B02.7). Malignant neoplasm of choroid (ICD-10-CM C69.3) and benign paroxysmal vertigo (ICD-10-CM H81.10) were selected as a negative control outcome, given its expected lack of association with zoster vaccination.

2.6. Statistical analysis

Patients with a history of the outcome of interest before the start of the follow-up window were excluded. Follow-up was censored at the earliest of the following: (1) occurrence of the outcome of interest in the patient's EHR, (2) the last recorded entry in the patient's EHR, or (3) the end of the follow-up window. Kaplan-Meier and Cox proportional hazards models were used to assess risk. Statistical significance was defined as a log-rank test $p < 0.05$ and a proportionality test $p > 0.01$, both implemented within the TriNetX platform. Hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated for each outcome. These analyses were performed using the survival package in R (version 3.2-3)

and validated against SAS (version 9.4).

3. Results

3.1. Shingrix vaccination vs presbyopia controls

Before PSM, the study population included 818,354 patients in the Shingrix vaccination group and 863,894 patients in the presbyopia control group with mean ages of 64.44 ± 8.9 years and 63.52 ± 9.14 years, respectively. Compared with controls, the Shingrix group was older and had higher proportions of White and Asian patients and lower proportions of Black or African American patients. The Shingrix group had higher rates of several systemic comorbidities and systemic corticosteroid use, and lower rates of ocular comorbidities, ophthalmic procedures and diabetes mellitus. All differences were statistically significant ($P < 0.05$) and demonstrated a standardized mean difference >0.1 . After 1:1 PSM, both groups included 571,746 patients, and all baseline characteristics were well balanced. The mean follow-up time was 3.54 ± 1.51 years and 3.27 ± 1.85 years, with median follow-up durations of 3.95 and 3.97 years, respectively. The characteristics of both cohorts, before and after PSM, are presented in Table 1.

At 5 years, 67 Shingrix vaccinated patients and 162 presbyopia

control patients developed zoster keratitis, with a HR of 0.50 (0.012% vs 0.028%; 95% CI [0.37, 0.67]; $p < 0.0001$). Zoster conjunctivitis was also less common in the Shingrix group, occurring in 34 patients compared with 78 patients in the control group (0.006% vs 0.014%; HR, 0.53; 95% CI [0.35, 0.81]; $p = 0.0025$).

As expected, positive control outcomes were less common in the treatment group. VZV infection with CNS involvement occurred in 1232 patients in the treatment group and 2915 patients in the control group, corresponding to a HR of 0.49 (0.217% vs 0.512%; 95% CI [0.46, 0.52]; $p < 0.0001$). Disseminated zoster infection occurred in 109 patients in the treatment group and 230 patients in the control group, corresponding to a HR of 0.59 (0.019% vs 0.040%; 95% CI [0.47, 0.75]; $p < 0.0001$). Malignant neoplasm of choroid and benign paroxysmal vertigo (BPPV), used as negative control outcomes, were comparable between groups with HRs close to 1. These results are presented in Fig. 1 and Table 2.

3.2. Shingrix vaccination vs quadrivalent influenza vaccination

Before PSM, the study population included 818,354 patients in the Shingrix vaccination group and 1,053,122 patients in the influenza vaccination control group with mean ages of 64.44 ± 8.9 years and

Table 1

Baseline characteristics of Shingrix-vaccinated patients and presbyopia controls presented before and after propensity score matching (PSM).

Characteristic Name	Before PSM				After PSM			
	Shingrix (n = 818,354)	Presbyopia (n = 863,894)	P	Std diff.	Shingrix (n = 571,746)	Presbyopia (n = 571,746)	P	Std diff.
Age at Index (mean $\pm$ SD)	64.44 $\pm$ 8.9	63.52 $\pm$ 9.14	<0.0001	0.10	64.24 $\pm$ 8.86	64.46 $\pm$ 8.93	<0.0001	0.03
White (%)	660094 (80.66)	585559 (68.97)	<0.0001	0.27	445909 (77.99)	445387 (77.9)	0.2391	0.00
Female (%)	467886 (57.17)	489234 (57.63)	<0.0001	0.01	326734 (57.15)	327283 (57.24)	0.2995	0.00
Male (%)	350296 (42.8)	359684 (42.37)	<0.0001	0.01	244942 (42.84)	244402 (42.75)	0.3074	0.00
Black or African American (%)	58535 (7.15)	113294 (13.34)	<0.0001	0.21	52099 (9.11)	54804 (9.59)	<0.0001	0.02
Hispanic or Latino (%)	39528 (4.83)	53914 (6.35)	<0.0001	0.07	30970 (5.42)	32388 (5.66)	<0.0001	0.01
Asian (%)	44305 (5.41)	24299 (2.86)	<0.0001	0.13	21563 (3.77)	21112 (3.69)	0.0261	0.00
Endocrine, nutritional and metabolic diseases (%)	562570 (68.74)	512288 (60.34)	<0.0001	0.18	359604 (62.9)	364267 (63.71)	<0.0001	0.02
Diseases of the musculoskeletal system and connective tissue (%)	541678 (66.19)	517469 (60.95)	<0.0001	0.11	354780 (62.05)	357671 (62.56)	<0.0001	0.01
Hypertensive diseases (%)	381127 (46.57)	372576 (43.88)	<0.0001	0.05	252763 (44.21)	257023 (44.95)	<0.0001	0.01
Hyperlipidemia, unspecified (%)	323276 (39.5)	283619 (33.41)	<0.0001	0.13	204723 (35.81)	208050 (36.39)	<0.0001	0.01
Cholesterol in LDL [Mass/volume] in serum or plasma	103 $\pm$ 35.9	104 $\pm$ 37.6	<0.0001	0.03	102 $\pm$ 36	104 $\pm$ 37.5	<0.0001	0.06
Cholesterol in HDL [Mass/volume] in serum or plasma	54.9 $\pm$ 18.6	49.4 $\pm$ 20.4	<0.0001	0.28	54.6 $\pm$ 18.7	49.2 $\pm$ 20.8	<0.0001	0.28
Mental, Behavioral and Neurodevelopmental disorders (%)	300655 (36.74)	292209 (34.42)	<0.0001	0.05	199357 (34.87)	202015 (35.33)	<0.0001	0.01
Diseases of the eye and adnexa (%)	214498 (26.21)	395375 (46.57)	<0.0001	0.43	191458 (33.49)	192668 (33.7)	0.0166	0.00
Overweight and obesity (%)	179379 (21.92)	160895 (18.95)	<0.0001	0.07	113438 (19.84)	115369 (20.18)	<0.0001	0.01
Diabetes mellitus (%)	145219 (17.74)	198294 (23.36)	<0.0001	0.14	113666 (19.88)	117861 (20.61)	<0.0001	0.02
Hemoglobin A1c/hemoglobin total in Blood	6.0 $\pm$ 1.12	6.46 $\pm$ 1.62	<0.0001	0.33	6.11 $\pm$ 1.21	6.36 $\pm$ 1.52	<0.0001	0.18
Ischemic heart diseases (%)	106891 (13.06)	105797 (12.46)	<0.0001	0.02	72332 (12.65)	73351 (12.83)	0.0043	0.01
Chronic kidney disease (%)	60850 (7.44)	64476 (7.6)	0.0001	0.01	42219 (7.38)	43194 (7.55)	0.0005	0.01
Dry eye syndrome (%)	35676 (4.36)	84914 (10.0)	<0.0001	0.22	34085 (5.96)	35587 (6.22)	<0.0001	0.01
Other disorders of cornea (%)	8753 (1.07)	19667 (2.32)	<0.0001	0.10	8365 (1.46)	8471 (1.48)	0.4105	0.00
Tobacco use (%)	24084 (2.94)	29215 (3.44)	<0.0001	0.03	17879 (3.13)	18431 (3.22)	0.0032	0.01
Alcohol related disorders (%)	24081 (2.94)	30194 (3.56)	<0.0001	0.03	17781 (3.11)	18312 (3.2)	0.0045	0.01
Immunodeficiency, unspecified (%)	8493 (1.04)	5776 (0.68)	<0.0001	0.04	4808 (0.84)	4633 (0.81)	0.0705	0.00
Human immunodeficiency virus [HIV] disease (%)	5586 (0.68)	4249 (0.5)	<0.0001	0.02	3340 (0.58)	3251 (0.57)	0.2716	0.00
Malignant neoplasms of lymphoid, hematopoietic and related tissue (%)	15481 (1.89)	11331 (1.34)	<0.0001	0.04	8983 (1.57)	8873 (1.55)	0.4067	0.00
Malignant (primary) neoplasm, unspecified (%)	11792 (1.44)	8758 (1.03)	<0.0001	0.04	6895 (1.21)	6860 (1.2)	0.7640	0.00
Corticosteroids for systemic use (%)	418164 (51.1)	340080 (40.06)	<0.0001	0.22	249611 (43.66)	249188 (43.58)	0.4251	0.00
Immunosuppressants (%)	42636 (5.21)	36577 (4.31)	<0.0001	0.04	26385 (4.62)	26226 (4.59)	0.4779	0.00
Oncology Treatments (%)	114118 (13.94)	97215 (11.45)	<0.0001	0.07	70623 (12.35)	70471 (12.33)	0.6656	0.00
Ophthalmology Services and Procedures (%)	68248 (8.34)	182842 (21.54)	<0.0001	0.38	66694 (11.66)	68984 (12.06)	<0.0001	0.01
Surgical Procedures on the Eye and Ocular Adnexa (%)	27209 (3.32)	53274 (6.28)	<0.0001	0.14	24674 (4.32)	25056 (4.38)	0.0799	0.00
Visit: Ambulatory (%)	659612 (80.6)	692995 (81.63)	<0.0001	0.03	448639 (78.47)	449236 (78.57)	0.1740	0.00
Visit: Emergency (%)	201565 (24.63)	232458 (27.38)	<0.0001	0.06	142635 (24.95)	144547 (25.28)	<0.0001	0.01

PSM = propensity score matching; Std diff. = standardized mean difference.

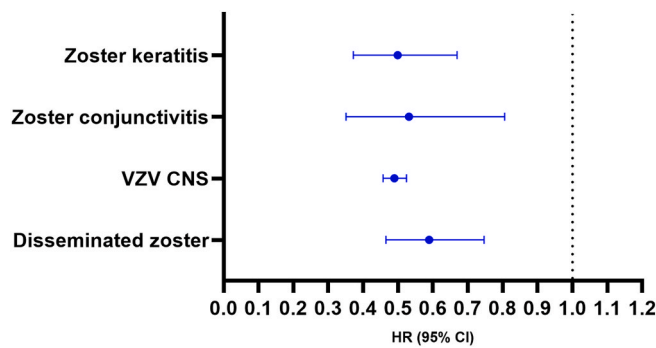


Fig. 1. Hazard ratios for zoster keratitis, zoster conjunctivitis, varicella-zoster virus infection with central nervous system involvement (VZV CNS) and disseminated zoster infection comparing Shingrix-vaccinated patients and presbyopia controls over a 5-year follow-up period beginning 30 days after the index event.

60.01 ± 7.62 years, respectively. Compared with controls, the Shingrix group was older and had higher proportions of White patients and lower proportions of Black or African American patients. Overall, patients in the Shingrix group demonstrated lower rates of several systemic comorbidities. After 1:1 PSM, both groups included 626,697 patients, and all baseline characteristics were well balanced. The mean follow-up time was 3.46 ± 1.53 years and 3.81 ± 1.54 years, with median follow-up durations of 3.82 and 4.96 years, respectively. The characteristics of both cohorts, before and after PSM, are presented in Table 3.

At 5 years, zoster keratitis developed in 45 Shingrix-vaccinated patients and 89 influenza-vaccinated control patients, corresponding to a HR of 0.56 (0.007% vs 0.014%; 95% CI [0.39, 0.81]; $p = 0.0016$). Zoster conjunctivitis was also less common in the Shingrix group, occurring in 28 patients compared with 84 patients in the control group (0.004% vs 0.013%; HR, 0.37; 95% CI [0.24, 0.57]; $p < 0.0001$).

As expected, positive control outcomes were less common in the treatment group, whereas negative control outcomes were comparable between groups, with HRs close to 1. These results are presented in Fig. 2 and Table 4.

4. Discussion

In this large, multicenter cohort study, administration of the Shingrix

vaccine among individuals without prior zoster ocular disease was associated with a reduced risk of VZV keratitis and VZV conjunctivitis. These associations remained robust in analyses comparing Shingrix recipients with propensity score matched presbyopia controls, used as a proxy for access to ophthalmic care in adulthood, as well as with individuals vaccinated with the seasonal quadrivalent influenza vaccine. Cohort validity was assessed using positive and negative control outcomes. As expected, rates of disseminated zoster infection and zoster with CNS involvement were significantly lower in the Shingrix vaccinated group. In contrast, no meaningful differences were observed for the negative control outcomes.

VZV keratitis is a potentially sight-threatening manifestation of HZO and has been reported to result in permanent corneal scarring in approximately 15% of affected patients [13]. The recombinant zoster vaccine (Shingrix) is highly effective in preventing a first episode of HZO, with an estimated effectiveness of 73%–89% [8,14]. However, no study has yet evaluated whether, and to what extent, the vaccination specifically reduces the risk of this severe ocular complication. This study is the first to evaluate the effectiveness of Shingrix vaccination in preventing zoster ocular surface complications, demonstrating a 44%–50% reduction in the risk of zoster keratitis and a 47%–63% reduction in the risk of zoster conjunctivitis.

The absolute risk reductions associated with Shingrix vaccination were modest, likely reflecting the relative rarity of zoster keratitis in the general population. When compared with presbyopia controls, the 5-year absolute risk reduction was approximately 0.0166%, corresponding to a number needed to treat (NNT) of approximately 6000 to prevent one case of zoster keratitis. In comparison with influenza-vaccinated controls, the absolute risk reduction was 0.0070%, corresponding to an NNT of approximately 14,300. Importantly, Shingrix vaccination is administered with the primary goal of preventing herpes zoster systemic and neurologic complications [10]. Accordingly, the observed reduction in zoster keratitis should be interpreted as an additional benefit of a vaccine that is already widely recommended and implemented in adults aged ≥50 years. Moreover, given the broad population for whom vaccination is recommended, even modest absolute risk reductions may translate into large numbers at the population level.

However, the potential benefits of the vaccine remain underrecognized among ophthalmologists. In a survey from Spain, 27% of ophthalmology professionals were unaware that vaccines against herpes zoster exist, and 71% did not know the clinical indications for their use [15]. These findings may help raise awareness among ophthalmologists

Table 2

Summary of outcomes of interest comparing Shingrix-vaccinated patients with presbyopia controls over a 5-year follow-up period beginning 30 days after the index event.

Outcome	Patients in cohort		Patients with outcome		Incident risk (%)		Survival probability at the end of time window		HR [95% CI]	Log-rank test p-value	Proportionality test p-value
	Shingrix	Presbyopia	Shingrix	Presbyopia	Shingrix	Presbyopia	Shingrix	Presbyopia			
Zoster keratitis	571745	571745	67	162	0.0117	0.0283	0.9997	0.9993	0.5 [0.37, 0.67]	<0.0001	0.4168
Zoster conjunctivitis	571744	571746	34	78	0.0059	0.0136	0.9998	0.9997	0.53 [0.35, 0.81]	0.0025	0.4228
VZV CNS	567982	568917	1232	2915	0.2169	0.5124	0.9956	0.9883	0.49 [0.46, 0.52]	<0.0001	0.0289
Disseminated zoster	571510	571615	109	230	0.0191	0.0402	0.9992	0.9990	0.59 [0.47, 0.75]	<0.0001	0.0104
Malignant neoplasm of choroid	571625	571546	82	106	0.0143	0.0185	0.9997	0.9996	0.89 [0.66, 1.2]	0.433	0.0803
BPPV	562293	563361	8323	9490	1.4802	1.6845	0.9549	0.9616	1.04 [1.01, 1.07]	0.0129	<0.0001

HR = hazard ratio; VZV CNS = varicella-zoster virus central nervous system involvement; BPPV = benign paroxysmal positional vertigo.

Table 3

Baseline characteristics of Shingrix-vaccinated patients and quadrivalent influenza-vaccinated presented before and after propensity score matching (PSM).

Characteristic Name	Before PSM		<i>P</i>	Std diff.	After PSM		<i>P</i>	Std diff.
	Shingrix (n = 818,354)	Influenza (n = 1,053,122)			Shingrix (n = 626,697)	Influenza (n = 626,697)		
Age at Index (mean ± SD)	64.44 ± 8.9	60.01 ± 7.62	<0.0001	0.53	62.36 ± 8.14	62.16 ± 8.22	<0.0001	0.02
White (%)	660094 (80.66)	792508 (75.25)	<0.0001	0.13	501291 (79.99)	501204 (79.97)	0.8460	0.00
Female (%)	467886 (57.17)	576837 (54.77)	<0.0001	0.05	355497 (56.72)	358795 (57.25)	<0.0001	0.01
Male (%)	350296 (42.8)	476041 (45.2)	<0.0001	0.05	271076 (43.26)	267742 (42.72)	<0.0001	0.01
Black or African American (%)	58535 (7.15)	131155 (12.45)	<0.0001	0.18	53493 (8.54)	53895 (8.6)	0.1995	0.00
Hispanic or Latino (%)	39528 (4.83)	51699 (4.91)	0.0129	0.00	30452 (4.86)	30255 (4.83)	0.4124	0.00
Asian (%)	44305 (5.41)	36954 (3.51)	<0.0001	0.09	27939 (4.46)	28175 (4.5)	0.3080	0.00
Endocrine, nutritional and metabolic diseases (%)	562570 (68.74)	797797 (75.76)	<0.0001	0.16	464028 (74.04)	464405 (74.1)	0.4422	0.00
Diseases of the musculoskeletal system and connective tissue (%)	541678 (66.19)	759689 (72.14)	<0.0001	0.13	444899 (70.99)	443199 (70.72)	0.0008	0.01
Hypertensive diseases (%)	381127 (46.57)	582128 (55.28)	<0.0001	0.17	318168 (50.77)	316168 (50.45)	0.0004	0.01
Hyperlipidemia, unspecified (%)	323276 (39.5)	456775 (43.37)	<0.0001	0.08	265673 (42.39)	263678 (42.07)	0.0003	0.01
Cholesterol in LDL [Mass/volume] in serum or plasma	103 ± 35.8	105 ± 37.8	<0.0001	0.06	104 ± 36.1	105 ± 37.3	<0.0001	0.05
Cholesterol in HDL [Mass/volume] in serum or plasma	54.9 ± 18.6	49.8 ± 20.7	<0.0001	0.26	54.6 ± 18.6	50.7 ± 20.9	<0.0001	0.20
Mental, Behavioral and Neurodevelopmental disorders (%)	300655 (36.74)	515864 (48.98)	<0.0001	0.25	264493 (42.2)	264495 (42.2)	0.9971	0.00
Diseases of the eye and adnexa (%)	214498 (26.21)	300617 (28.55)	<0.0001	0.05	172352 (27.5)	168164 (26.83)	<0.0001	0.02
Overweight and obesity (%)	179379 (21.92)	277785 (26.38)	<0.0001	0.10	154366 (24.63)	155400 (24.8)	0.0323	0.00
Diabetes mellitus (%)	145219 (17.74)	258771 (24.57)	<0.0001	0.17	127921 (20.41)	126480 (20.18)	0.0014	0.01
Hemoglobin A1c/hemoglobin total in Blood	6.0 ± 1.12	6.32 ± 1.59	<0.0001	0.23	6.03 ± 1.16	6.15 ± 1.42	<0.0001	0.10
Ischemic heart diseases (%)	106891 (13.06)	156737 (14.88)	<0.0001	0.05	87272 (13.93)	85297 (13.61)	<0.0001	0.01
Chronic kidney disease (%)	60850 (7.44)	84529 (8.03)	<0.0001	0.02	48490 (7.74)	47085 (7.51)	<0.0001	0.01
Dry eye syndrome (%)	35676 (4.36)	44196 (4.2)	<0.0001	0.01	27616 (4.41)	26663 (4.26)	<0.0001	0.01
Other disorders of cornea (%)	8753 (1.07)	9610 (0.91)	<0.0001	0.02	6373 (1.02)	6032 (0.96)	0.0021	0.01
Tobacco use (%)	24084 (2.94)	74395 (7.06)	<0.0001	0.19	23677 (3.78)	23723 (3.78)	0.8295	0.00
Alcohol related disorders (%)	24081 (2.94)	56554 (5.37)	<0.0001	0.12	22511 (3.59)	22025 (3.51)	0.0190	0.00
Immunodeficiency, unspecified (%)	8493 (1.04)	8701 (0.83)	<0.0001	0.02	6303 (1.01)	5979 (0.95)	0.0033	0.01
Human immunodeficiency virus [HIV] disease (%)	5586 (0.68)	11391 (1.08)	<0.0001	0.04	5065 (0.81)	4606 (0.74)	<0.0001	0.01
Malignant neoplasms of lymphoid, hematopoietic and related tissue (%)	15481 (1.89)	19288 (1.83)	0.0025	0.00	12015 (1.92)	11288 (1.8)	<0.0001	0.01
Malignant (primary) neoplasm, unspecified (%)	11792 (1.44)	12665 (1.2)	<0.0001	0.02	8741 (1.4)	8179 (1.3)	<0.0001	0.01
Corticosteroids for systemic use (%)	418164 (51.1)	563236 (53.48)	<0.0001	0.05	339981 (54.25)	337342 (53.83)	<0.0001	0.01
Immunosuppressants (%)	42636 (5.21)	52213 (4.96)	<0.0001	0.01	32698 (5.22)	31178 (4.97)	<0.0001	0.01
Oncology Treatments (%)	114118 (13.94)	136012 (12.92)	<0.0001	0.03	87274 (13.93)	85354 (13.62)	<0.0001	0.01
Ophthalmology Services and Procedures (%)	68248 (8.34)	126907 (12.05)	<0.0001	0.12	59975 (9.57)	57797 (9.22)	<0.0001	0.01
Surgical Procedures on the Eye and Ocular Adnexa (%)	27209 (3.32)	30400 (2.89)	<0.0001	0.03	20192 (3.22)	19270 (3.08)	<0.0001	0.01
Visit: Ambulatory (%)	659612 (80.6)	990948 (94.1)	<0.0001	0.41	570952 (91.1)	566401 (90.38)	<0.0001	0.03
Visit: Emergency (%)	201565 (24.63)	365605 (34.72)	<0.0001	0.22	181460 (28.96)	184196 (29.39)	<0.0001	0.01

PSM = propensity score matching; Std diff. = standardized mean difference.

and cornea specialists and support a more active role in the prevention of zoster keratitis, particularly in high-risk populations. Such populations primarily include older adults and immunocompromised

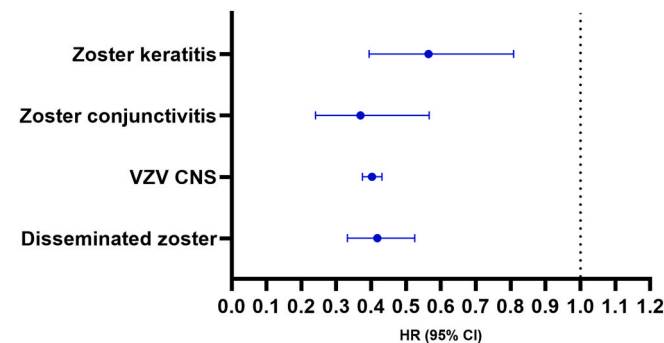


Fig. 2. Hazard ratios for zoster keratitis, zoster conjunctivitis, varicella-zoster virus infection with central nervous system involvement (VZV CNS) and disseminated zoster infection comparing Shingrix-vaccinated patients and quadrivalent influenza-vaccinated controls over a 5-year follow-up period beginning 30 days after the index event.

patients, such as recipients of hematopoietic cell or solid organ transplantation, individuals with malignancy or HIV infection, and those receiving systemic immunosuppressive therapies, including corticosteroids [16,17].

Greater vaccination rates, in turn, will reduce long-term healthcare utilization and ease the burden on tertiary corneal services. Preventing zoster keratitis may also carry meaningful economic benefits. Recurrent herpes dendritiform keratitis, chronic stromal inflammation, neurotrophic ulcers, and post-infectious neovascularization and scarring often necessitate multiple clinic visits, long-term topical therapy, repeated imaging, and, in advanced cases, surgical interventions [6]. These treatments are resource-intensive and costly, and by reducing the incidence of these complications, Shingrix vaccination has the potential to lessen the economic burden on both patients and healthcare systems. This may be particularly relevant in resource-limited settings, where access to ophthalmic care is restricted. In addition, vaccination may reduce population-level impairment in quality of life, as recurrent inflammatory episodes are frequently associated with significant ocular pain and photophobia and may lead to permanent visual impairment [2, 4–6]. These chronic and relapsing manifestations may interfere with daily functioning and necessitate prolonged engagement with ophthalmic care. In this context, the Zoster Eye Disease Study (ZEDS)

Table 4

Summary of outcomes of interest in Shingrix-vaccinated patients versus quadrivalent influenza-vaccinated controls over a 5-year follow-up period beginning 30 days after the index event.

Outcome	Patients in cohort		Patients with outcome		Incident risk (%)		Survival probability at the end of time window		HR [95% CI]	Log-rank test p-value	Proportionality test p-value
	Shingrix	Influenza	Shingrix	Influenza	Shingrix	Influenza	Shingrix	Influenza			
Zoster keratitis	626696	626694	45	89	0.0072	0.0142	0.9999	0.9998	0.56 [0.39, 0.81]	0.0016	0.2117
Zoster conjunctivitis	626696	626695	28	84	0.0045	0.0134	0.9999	0.9998	0.37 [0.24, 0.57]	<0.0001	0.1504
VZV CNS	622346	623032	1104	3022	0.1774	0.4850	0.9974	0.9937	0.4 [0.38, 0.43]	<0.0001	0.2552
Disseminated zoster	626394	626584	101	269	0.0161	0.0429	0.9998	0.9994	0.42 [0.33, 0.52]	<0.0001	0.4788
Malignant neoplasm of choroid	626586	626588	64	60	0.0102	0.0096	0.9998	0.9999	1.2 [0.84, 1.7]	0.3218	0.4497
BPPV	615274	618071	8034	8948	1.3058	1.4477	0.9806	0.9810	1.0 [0.97, 1.03]	0.8723	<0.0001

HR = hazard ratio; VZV CNS = varicella-zoster virus central nervous system involvement; BPPV = benign paroxysmal positional vertigo.

demonstrated that preventive daily valacyclovir at a dose of 1000 mg was associated with a significant reduction in the risk of new or worsening zoster keratitis or iritis at the 18-month endpoint, as well as a reduced incidence of multiple recurrent episodes at both 12 and 18 months [18]. While these findings support consideration of long-term suppressive antiviral therapy, they also underscore the substantial disease burden, as prolonged treatment, potentially associated with adverse effects, may be required and recurrence can still occur despite therapy. Taken together, these observations highlight the value of primary preventive strategies, such as vaccination, aimed at reducing the initial occurrence of zoster keratitis.

This study has several important strengths. To the best of our knowledge, it is the first study to evaluate whether, and to what extent, Shingrix vaccination reduces the specific risk of zoster keratitis. The large cohort size enabled the detection of statistically significant risk reductions, despite the modest absolute risk reduction observed. In addition, although beyond the primary scope of this study, our findings also demonstrated that Shingrix vaccination was associated with a reduced risk of CNS involvement and disseminated zoster infection, further supporting the broader protective effects of vaccination.

However, this study also has several limitations that should be acknowledged. First, its retrospective design and reliance on EHR data introduce potential sources of bias, including variability in diagnostic coding, incomplete clinical documentation, and loss to follow-up. To address these limitations, we incorporated both positive and negative control outcomes. As expected, vaccine-preventable systemic zoster complications were significantly reduced in the vaccinated cohort, whereas outcomes unrelated to vaccination demonstrated HRs close to unity. This pattern supports the internal validity of the analysis and reduces concern for systematic outcome-assessment bias inherent to EHR-based studies. Second, although extensive PSM was performed, and an active comparator cohort of influenza-vaccinated patients was used to address potential differences in healthcare access and health-seeking behavior, residual confounding cannot be excluded. Third, outcomes were assessed within a 5-year follow-up window, as longer-term follow-up was limited by data availability. Fourth, the assessment of keratitis-related complications was restricted, as many clinically relevant endpoints lack condition-specific diagnostic codes. Finally, detailed information regarding clinical phenotype (e.g., epithelial versus stromal involvement), disease severity and duration, lesion location, and

associated visual outcomes was unavailable, as such data are not captured within the TriNetX platform. Nevertheless, our conclusions are based on time-to-event analyses using HRs to quantify the association between vaccination and incident zoster keratitis rather than on granular phenotypic characterization, and our interpretation is limited to this scope.

Our findings may motivate future studies incorporating more granular clinical data to characterize the effects of vaccination on disease phenotype, severity, duration, and visual outcomes, thereby expanding understanding of its protective role on the ocular surface. In addition, dedicated cost-effectiveness analyses that incorporate ophthalmic outcomes represent another important direction for future research.

5. Conclusions

In this large, multicenter cohort study, adult recombinant zoster vaccination was, for the first time, associated with a significantly reduced risk of first-episode zoster keratitis. These findings support a more active role for ophthalmologists in the prevention of zoster keratitis, particularly among high-risk populations, and highlight the potential population-level and economic benefits of increased vaccination uptake, especially in resource-limited settings.

CRediT authorship contribution statement

Yossi Eshel: Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Natan Lishinsky-Fischer:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Itay Nitzan:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Nir Erdinest:** Writing – review & editing, Writing – original draft, Formal analysis. **Shoham Kubovsky:** Writing – review & editing, Methodology, Formal analysis. **Denise Wajnsztajn:** Writing – review & editing, Methodology, Formal analysis. **Jaime Levy:** Writing – review & editing, Methodology, Formal analysis. **Zvi Gur:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Itay Lavy:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

Data statement

This study used de-identified data from the TriNetX Research Network. Access to these data is restricted by TriNetX data use agreements and cannot be shared publicly. Investigators with appropriate permissions and access to TriNetX may replicate the analyses using the methodology described.

Disclosure

The authors declare no competing interests.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this manuscript, the authors used an AI-based language model (ChatGPT) to assist with language editing. All content was subsequently reviewed and edited by the authors, who take full responsibility for the accuracy and integrity of the work.

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Glossary

Varicella-Zoster Virus (VZV) A neurotropic human herpesvirus that causes primary infection as varicella (chickenpox) and establishes lifelong latency in sensory dorsal root and cranial nerve ganglia. Reactivation later in life results in herpes zoster (shingles)

Herpes zoster ophthalmicus (HZO) Reactivation of VZV involving the ophthalmic (V1) branch of the trigeminal nerve, often associated with ocular and periocular involvement and potentially sight-threatening complications. Nearly any ocular structure may be affected

Herpes zoster keratitis VZV infection involving the corneal epithelium, stroma, and/or endothelium. Clinical manifestations range from acute dendriform keratitis to chronic stromal keratitis and neurotrophic keratopathy and may result in corneal scarring and significant vision loss

Herpes zoster conjunctivitis Inflammation of the conjunctiva associated with herpes zoster ophthalmicus

Recombinant zoster vaccine (Shingrix; VZV glycoprotein E) A non-live, recombinant, adjuvanted vaccine containing VZV glycoprotein E, approved for the prevention of herpes zoster and its complications in adults aged ≥ 50 years and in immunocompromised individuals aged ≥ 18 years

Influenza quadrivalent vaccine A seasonal influenza vaccine containing antigens from four influenza virus strains, administered annually to prevent seasonal influenza. It is available in multiple formulations, including inactivated, recombinant, and, in some settings, adjuvanted vaccines

TriNetX A global federated electronic health record (EHR) research network providing real-world clinical data for multi-institutional observational studies

Electronic health record (EHR) A digital medical record containing patient demographic, clinical, and encounter-level health information used for large-scale clinical research

Propensity score matching (PSM) A statistical technique used to balance baseline characteristics between groups in observational studies to reduce confounding

Index event The predefined starting point for follow-up in a cohort study, typically the date of exposure or diagnosis from which outcomes are measured

Positive control outcomes Outcomes known or expected to be associated with the exposure of interest, used to assess cohort validity and confirm that the study design can detect true associations

Negative control outcomes Outcomes not expected to be associated with the exposure of interest, used to evaluate residual confounding and detect potential bias

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