

Meganuclease targeting HSV-1 protects against herpetic keratitis: Application to corneal transplants

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Herpes simplex virus (HSV) infection is a leading cause of corneal blindness. However, keratoplasty is only rarely proposed due to the high frequency of graft failure and associated recurrences. Gene therapy of the corneal graft might provide sustained protection against HSV infection. To test that hypothesis, we designed a meganuclease specific to an HSV-1 DNA sequence coding for major capsid protein (UL19) and selected an adeno-associated virus type-2 as the vector. Meganuclease was transduced into corneas and its effect was challenged *in vitro*, *ex vivo*, and then *in vivo* in a rabbit HSV-1-infection model of stromal keratitis and endotheliitis. *In vivo*, meganuclease exposure resulted in fewer infected stromal and endothelial cells, and protected against corneal opacification and edema. *Ex vivo*, HSV-1 infection rates of meganuclease-treated human corneas were drastically reduced. Furthermore, genetically engineered corneas transplanted *in vivo* into rabbit eyes protected against HSV-1 infection. This genome-editing technology targeting HSV-1 opens new opportunities to manage severe post-herpetic corneal blindness by providing infected patients with genetically protected corneal transplants.

INTRODUCTION

Among corneal diseases leading to blindness, herpes simplex keratitis (HSK) is the most frequent in the United States and in western Europe.¹ The global HSK incidence is roughly 1.5 million, including 40,000 new cases of severe monocular visual impairment or blindness each year. While epithelial lesions are the most frequent, stromal and endothelial HSK infections are the most severe, leading to corneal edema and/or scarring, due to the direct pathogenic effect of the virus and the intensity of the host immune response against the infected tissue.² While patients can receive a corneal graft, its prognosis is poor, partly because of HSK recurrence, which requires lifelong antiviral use.³ In addition, enhanced resistance is increasingly reported in immunocompromised and immunocompetent patients.^{4,5} Furthermore, acyclovir prophylaxis may predispose to antiviral resistant recurrent HSK.⁶

To obtain corneal protection against herpes simplex virus 1 (HSV-1), we developed a gene-therapy approach using a meganuclease,⁷ an endonuclease specifically recognizing HSV-1 DNA.⁸ The meganuclease was delivered *ex vivo* and *in vivo* to corneal endothelial or stromal cells via an adeno-associated virus (AAV) vector and tested in models of HSK that we developed. A rabbit model was used because of the corneal anatomy and the size close to the ones of the human eye, making possible the easy realization of surgical acts such as they could be practiced in the human within the framework of translational research. Eventually, we tested the protection conferred by the meganuclease in an *in vivo* model of corneal transplant.

RESULTS

Candidate vectors suitable for gene therapy were compared for their capacity to lead the expression of the enhanced green fluorescent protein (EGFP) in rabbit corneal endothelium (Figure S1). Among vectors derived from AAV type-2 (pseudotypes 1 and 5, e.g., AAV-2/1), the AAV-2/1 vector was retained for its transduction efficiency, its nonintegrative character,⁹ and the sustainability of the transgenic expression it induces.^{10,11} The AAV-EGFP vector achieved transfection of endothelial cells, as demonstrated by the detection of a fluorescent signal for up to 32 days posttransfection (Figure 1).

To monitor herpetic infection, we used a recombinant strain F that contains a β -galactosidase gene-expression cassette inserted into both latency-associated transcript loci (recombinant (r)HSV-1). We found that *in vitro* β -galactosidase activity and real-time quantitative polymerase chain reaction (qPCR)-determined viral genome (vg) numbers were correlated (Spearman's rank correlation [Rs], lowest Rs = 0.92 and highest two-tailed p < 0.001; Figure S2), thereby allowing use of the X-Gal-generated chromophore (henceforth, X-Gal-c) as a reporter to monitor viral infection. In an *ex vivo* model

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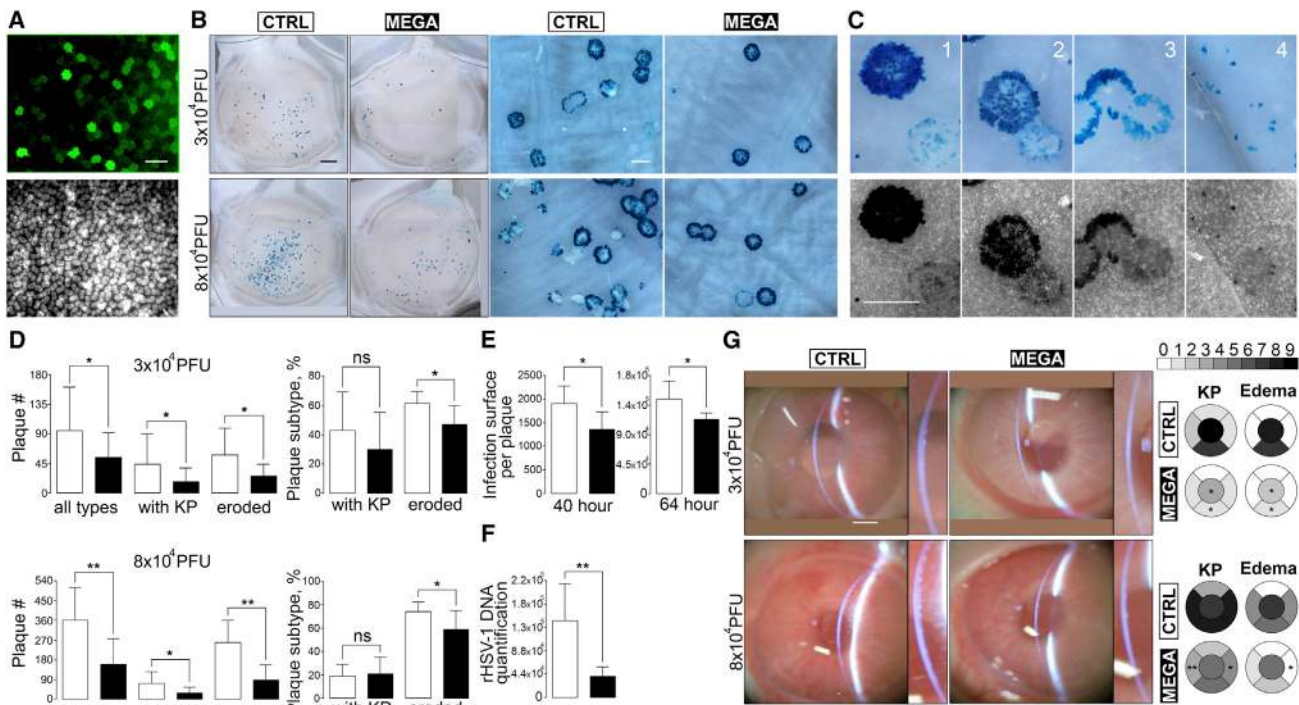


Figure 1. Meganuclease targeting HSV-1 protects against endothelial infection in an *in vivo* rabbit herpetic keratitis model

(A) *In vivo* endothelium transduction efficiency after intracameral adeno-associated virus-enhanced green fluorescent protein (AAV-EGFP) injection. Macroscopic examination for reporter direct fluorescence (top) and DAPI staining (bottom) 2 weeks after vector injection (n = 6). Scale bar, 50 μ m. (B–E) Analysis of endothelial X-Gal-generated chromophore (X-Gal-c) release 40 h after rHSV-1 infection. Two weeks after intracameral injection of AAV-control (AAV-CTRL)- or meganuclease (AAV-MEGA)-vector suspension, the anterior chamber was inoculated with 3×10^4 or 8×10^4 rHSV-1 PFU (four experiments in the 3×10^4 -PFU group samples n = 13; three experiments in the 8×10^4 -PFU group with nine controls and eight test samples). (B) Macroscopic endothelial X-Gal-c signals. X-Gal-c-positive clusters of infected endothelial cells (plaque) in AAV-CTRL- or AAV-MEGA-treated corneas. Scale bars, 1 mm (left) and 250 μ m (right). (C) Endothelial plaque-subtype analysis. X-Gal-c release (top) and DAPI staining (bottom). 1: endothelial plaque; 2: keratic precipitate (KP)-associated plaque; 3, 4: eroded plaques. Scale bar, 250 μ m. (D) Endothelial plaque and subtype quantification. ImageJ analysis using the outlines of endothelial X-Gal-c release in AAV-CTRL- (white columns) and AAV-MEGA-treated (black columns). Results are expressed as mean $\pm$ SD and percentage $\pm$ SD for representative experiments (n = 8 for each group). Two-sided Mann-Whitney test or Kolmogorov-Smirnov (D) test; *p < 0.05, **p < 0.01, ***p < 0.001. (E) Individual plaque-area analysis (including eroded plaques) after rHSV-1 infection (six control and test corneas of the first experiments). (F) qPCR rHSV-1 genome/quantification in the aqueous humor 40-h postinfection with 8×10^4 rHSV-1 PFU (eight controls and seven test eyes). Two-sided Mann-Whitney test: *p < 0.05, **p < 0.01, ***p < 0.001. (G) Corneal slit-lamp photographs postinfection with 3000 rHSV-1 PFU (top) or 8×10^4 PFU (bottom) showing broad and magnified representative views of endothelial KPs and focal corneal edema (scale bar, 2 mm). The presence of KPs and edema was scored 0 or 1 in each of the five corneal zones and their sums are represented schematically by sector (right). Fisher's exact test: *p < 0.05, **p < 0.01.

of endothelial HSK using rHSV-1 under agar, infected cells clustered into discoidal islets, called plaques (Figures S3 and S4), which were analyzed to assess meganuclease efficiency. Using this model, meganuclease targeting UL19 (HSV-1m2), the major viral capsid protein VP5 gene, provided better protection against HSV-1 than the infected-cell polypeptide-0 gene (ICP0; HSV-1m4) (Figure S5), and therefore was chosen and challenged in models of stromal or endothelial herpetic infection.

Meganuclease confers protection against herpetic endothelitis

Ex vivo, corneas transfected with the AAV-2/1 vector coding for HSV-1m2 meganuclease (AAV-MEGA) or a noncoding sequence (AAV-CTRL) were inoculated with rHSV-1 or SC16 (three experiments, n = 6 with 300 rHSV-1 plaque-forming units [PFUs]; two experiments, n = 8, with 300 or 600 SC16 PFUs) and endothelial

infection was analyzed 40 h later (Figures S6 and S7). The number of rHSV-1 particles inoculated into AAV-CTRL-injected corneas induced similar numbers of plaques after 40 h of incubation (six corneas, mean $\pm$ SD normalized to the endothelial area 296 ± 76), demonstrating highly efficient endothelial infection.

AAV-MEGA protected endothelial cells, with 56% to 64% significantly lower plaque numbers (two-sided Mann-Whitney test (henceforth Mann-Whitney) highest p = 0.005) and the 68% significantly lower X-Gal-c areas (one experiment; Mann-Whitney p = 0.005; Spearman's rank correlation between plaque number and X-Gal-c area, $R_s = 0.89$, p < 0.001). Local infection was also less, with a 47% drop in the number of infected cells per plaque in test samples (one experiment, six corneas, 50 plaques counted in zones of maximal endothelial density p = 0.008). AAV-MEGA also significantly limited plaque numbers

($p = 0.03$; Figure S7) in corneas infected with wild-type SC16: 56% smaller immunolabeled area (six of eight corneas infected with the lowest virus load (300 PFUs), normalized to the endothelial area; Mann-Whitney $p = 0.005$). The observed protection of the endothelium against infection was not related to potential cytotoxicity of the test vector, because SC16-infected controls (six for each condition) or test rHSV-1-infected samples (six for each condition) had similar endothelial cell densities and viability (CMRA fluorescence; Figure S7), suggesting the safety of meganuclease treatment.

In vivo, endothelial cell transduction after AAV-2/1-vector intracameral injection was sustained for at least 42 days postinoculation (Figure 1), enabling assessment of *in vivo* endothelial protection against rHSV-1. A herpetic endotheliitis model was created using partial immunosuppression, transient endothelial osmotic stress, and rHSV-1 intracameral injection (Figures S8 and S9). With this model, the formation of endothelial plaques, resembling those in the *ex vivo* model, and the appearance of typical clinical signs of endotheliitis were assessed after AAV-CTRL or AAV-MEGA pretreatment (two sets of experiments, $n = 3$ or 4 in each) and rHSV-1 inoculation 2 weeks later. Plaque numbers and diameters increased with virus load and time of inoculation. Plaque numbers reached means of ~ 100 (99 ± 66) and ~ 400 (362 ± 148) at 40 h after injecting 3×10^4 or 8×10^4 rHSV-1 PFUs (Figure 1D), respectively. Mean plaque diameter increased from $227 \pm 62 \mu\text{m}$ (40 h, 60 plaques in three corneas) to $415 \pm 66 \mu\text{m}$ (37 plaques in six corneas) 40 to 64 h after inoculating 3×10^4 PFUs (Figure S9). Interestingly, several plaque subtypes could be distinguished, some associated with inflammatory cells appearing as whitish keratic deposits overlying X-Gal-c-colored cells, other endothelial cell plaques had central erosions, devoid of cells, while others appeared intact without inflammation or erosions. Meganuclease pretreatment decreased overall plaque numbers for both 3×10^4 - and 8×10^4 -PFU inoculates by 43% and 56%, respectively (Mann-Whitney highest $p = 0.04$ for the 3×10^4 -PFU group; Figure 1D).

To determine the total number of cells infected, infected X-Gal-c-colored areas were added to eroded endothelial areas, which would be nucleus 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)- and X-Gal-c-negative within X-Gal-c-positive plaques for each injection site. The total infected areas per plaque were lower in AAV-MEGA-treated groups by $\sim 29\%$ and $\sim 25\%$ for ~ 40 -h and ~ 64 -h infections, respectively (Figure 1E, Mann-Whitney highest $p < 0.001$ at ~ 64 h), reflecting fewer infected cells. Ocular inflammation, observed 40 h postinoculation and resembling human herpetic endotheliitis, did not differ significantly between experimental groups, as assessed by aqueous humor protein and cell contents (Tyn-dall effect) ($n = 13$, Fisher's exact test [henceforth Fisher], $p > 0.05$).

Furthermore, in the in AAV-MEGA-treated groups slit-lamp assessment of keratic precipitates (KPs), corresponding to immune deposits on the infected endothelium, found fewer in the 3×10^4 - and 8×10^4 -PFU groups, respectively (Fisher $p = 0.03$ and 0.04) (Figure 1G). In addition, their respective macroscopically observed mean numbers of plaques associated with KPs were 63% and 60% lower (Mann-Whitney highest $p = 0.03$) (Figure 1D).

The AAV-MEGA vector also obtained significantly fewer mean numbers of eroded plaques, declining 66% and 79% in the 3×10^4 - and 8×10^4 -PFU groups, respectively (Mann-Whitney highest $p = 0.007$) (Figure 1D).

In this *in vivo* model, endotheliitis was associated with focal edema (Figure 1G), as in humans; the total numbers of edematous zones dropped from 15 to 3 and from 20 to 8 in 3×10^4 - and 8×10^4 -PFU-infected eyes, respectively (Fisher $p = 0.01$ and 0.04). This meganuclease protective effect against HSV-1 infection was corroborated by the 73% lower rHSV-1 qPCR-detected genome-copy numbers in aqueous humor (eight controls and seven test eyes for the 8×10^4 -PFU experiment, two measurements, Mann-Whitney $p = 0.02$; Figure 1F) that were correlated to plaque numbers ($R_s = 0.67$, highest $p = 0.004$).

AAV-MEGA pretreatment confers cornea-graft protection against HSV endotheliitis

Meganuclease protection of corneal endothelium was tested in a syngeneic cornea-graft model that was subsequently challenged with rHSV-1 infection. Rabbit corneal buttons were cultured with the AAV-MEGA or AAV-CTRL vector as described for *ex vivo* experiments; subsequently, the central part of the cornea was grafted into inbred animals. After healing, rHSV-1 was inoculated intracamerally, as in the *in vivo* model; plaque formation, virus-particle release into the anterior chamber, and corneal edema were analyzed 40 h later. Figure 2A shows representative photos of the entire grafted rabbit corneas, including the recipient bed and the transplant. The wound junction, delimited by red dots, shown at higher magnification in Figure 2B, was not analyzed. The mean number of plaques was 83 ± 39 (one out of the five samples was outside the standard deviation) (Figure 2C) in control-vector-pretreated transplants; it was $\sim 75\%$ lower for the AAV-MEGA-treated grafts (lower for each of the five eye pairs; Mann-Whitney $p = 0.01$). Plaque numbers on the transplants were also compared with those in the recipient bed, used as an internal control. Plaque numbers in AAV-CTRL-treated recipient beds were correlated to those in the unprotected transplant ($R_s = 0.9$, $p = 0.04$). The AAV-MEGA-treated transplant plaque numbers normalized to recipient bed plaque numbers was $\sim 70\%$ lower than in the AAV-CTRL-treated transplants (lower for each of the five eye pairs, Mann-Whitney $p = 0.01$). The numbers of KPs and eroded plaques on the AAV-MEGA-treated grafts were lower, respectively $\sim 86\%$ (lower for each of the five eye pairs Mann-Whitney $p = 0.01$; Figure 2C) and $\sim 84\%$ (Mann-Whitney $p = 0.04$). Importantly, no stromal edema was seen in any treated graft, but was present in four of five controls (Figure 2E, Fisher, $p = 0.047$). Viral particle numbers in aqueous humor of AAV-MEGA-transduced grafts were $\sim 64\%$ lower (Figure 2C, $n = 5$, Mann-Whitney $p = 0.04$).

AAV-MEGA efficiency against rHSV-1 endotheliitis in human corneas stored *ex vivo*

We then validated the AAV-MEGA-conferred protection of human corneas, as in the rabbit model. Paired human corneal buttons were exposed to AAV-CTRL or AAV-MEGA for 14 days in organ culture prior to rHSV-1 inoculation and analyzed 40 h later (Figure S10). Because the infection pattern differed in human and rabbit corneas,

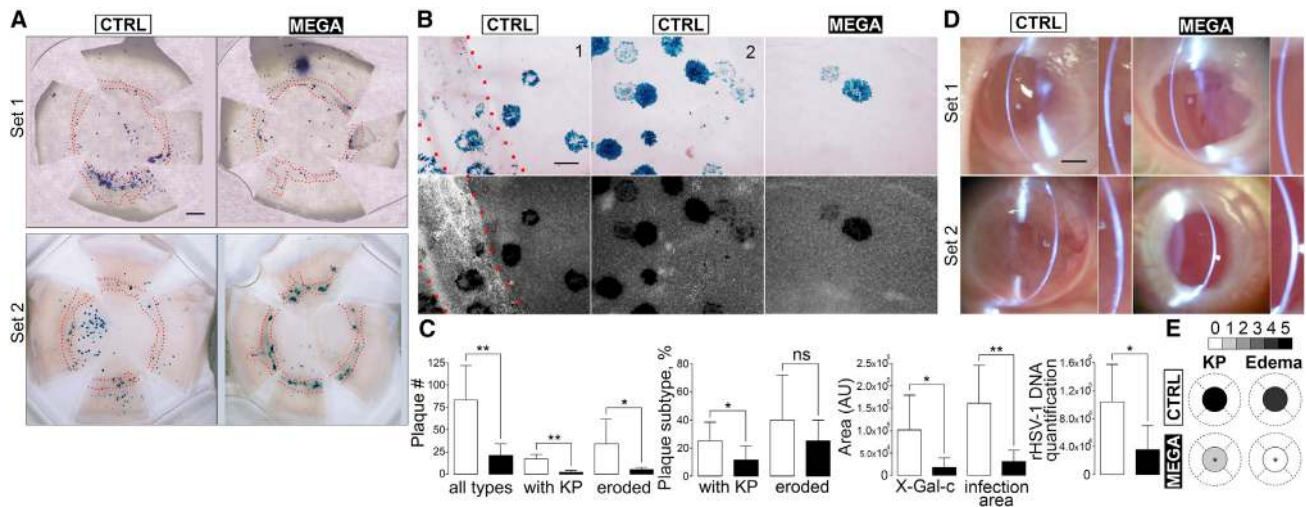


Figure 2. Meganuclease-treated corneal grafts are protected against rHSV-1 endothelial infection in an *in vivo* rabbit cornea-transplant model

Donor corneal buttons were cultured with adeno-associated-virus control (AAV-CTRL)- or meganuclease (AAV-MEGA)-coding vector. Recipient corneas were trephined and their central portion removed. Transduced donor corneas were trephined and sutured to the recipient corneal bed. Four weeks later, eyes were injected intracamerally with 9×10^4 rHSV-1 PFU. Corneal herpetic infections were analyzed 40 h after postinoculation ($n = 5$ in each group). (A and top panel B) Macroscopic image of endothelial X-Gal-generated chromophore (X-Gal-c) release. Scale bars, 2 mm (A) and 250 μ m (B). Dotted red lines represent graft suture lines containing fibrotic tissue. (B bottom panel) DAPI staining. (C) Endothelial plaque counts, subtype percentage, and total plaque-area analyses based on endothelial X-Gal-c release and qPCR determined rHSV-1 genome numbers. Infection area includes X-Gal-c-colored areas and eroded surfaces. Results are expressed as mean $\pm$ SD ($n = 5$ in each group). Two-sided Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) Slit-lamp photographs showing broad and magnified representative views of endothelial keratic precipitates (KPs) and focal corneal edema (scale bar, 2 mm). (E) Schematic diagrams of KP quantification and corneal edema ($n = 5$ in each group; Fisher's exact test; * $p < 0.05$).

with irregular and larger infection plaques, frequently reaching 800 μ m, in the control group (250 μ m in rabbit corneas), rendering plaque counting less appropriate, X-Gal-c area was used as the assessment index of rHSV-1 infection. Pertinently, human corneas had systematically lower infection levels than rabbit samples (2.89, 3.36, and 4.15 times weaker X-Gal-c signals for 20 $\times$ higher rabbit virus load, i.e., 6,000 rHSV-1 PFU), indicating lower permissiveness of human endothelium to HSV-1. Importantly, AAV-MEGA had an inhibitory effect on rHSV-1 infection, as demonstrated by $\sim 92\%$, 87%, and 75% weaker X-Gal-c signals for the highest virus load. Hence, AAV-MEGA treatment of stored human corneas was able to contain HSV-1 infection.

***In vivo* protection against stromal herpetic keratitis**

Because herpetic stromal keratitis is a leading cause of vision loss,¹² we assessed the meganuclease's therapeutic potential in rabbit corneal stroma. After confirming that stromal transduction followed intrastromal AAV-EGFP-vector injection and led to reporter expression (Figure 3A) and that AAV-MEGA-vector injection enabled meganuclease expression (Figure 3B), AAV-MEGA vector and rHSV-1 were injected sequentially following partial immunosuppression, known to exacerbate HSV-1 replication.^{13–15} Two weeks after stromal transfection of the AAV-CTRL vector, keratocyte infection was observed from day 1 to day 6 rHSV-1 postinoculation (Figure 3C).

On day 1, the numbers of X-Gal-c-colored keratocytes were close to the numbers of rHSV-1 particles injected (2,000 PFUs inoculated led to a mean of 1875 ± 662 infected cells for eight injection sites in a represen-

tative experiment), which suggests that the infection was in its first lytic cycle. On days 2 and 3, the numbers of infected keratocytes increased, respectively ~ 2.5 and ~ 4.6 times that of day 1 (six injection sites in each case). On day 6, because the infected keratocytes were too dense for individual counting, we used the X-Gal-c-colored area, after determining that the number of infected cells was indeed correlated to it ($R_s = 0.72$; highest $p = 0.001$). Pretreatment with AAV-MEGA vector led to 60% fewer infected keratocytes and smaller X-Gal-c area on day 1 (Figures 3C and 3D; three experiments with $n = 8$ injection sites; Mann-Whitney highest $p = 0.002$). On day 2, the numbers of X-Gal-c-colored keratocytes were $\sim 85\%$ lower ($n = 6$, Mann-Whitney $p = 0.005$); on day 6, AAV-MEGA treatment lowered the number by 90% (Figure 3D; two experiments with $n = 12$ injection sites; Mann-Whitney highest $p < 0.001$).

Stromal inoculation with rHSV-1 clinically reproduced the corneal inflammation observed in human stromal keratitis.¹⁶ In AAV-CTRL controls, stromal infiltrates, corresponding to clusters of infected keratocytes, were observed with slit lamp from day 3 (9 of 12 and 8 of 12 in two experiments, $n = 12$ injection spots) and were more visible on day 6 (11 of 12 and 10 of 12) (Figure 3E). Pretreatment with AAV-MEGA vector led to markedly less stromal inflammation (3 of 12 and 2 of 10 on day 3 or 6, respectively; Fisher lowest $p = 0.04$). Surprisingly, the intrastromal rHSV-1 injection subsequently spread to the epithelium (Figure 3F), with punctual epithelial lesions as of day 3, and number and size increased gradually over the following days. On day 6, epithelial X-Gal-c release was $\sim 63\%$ and

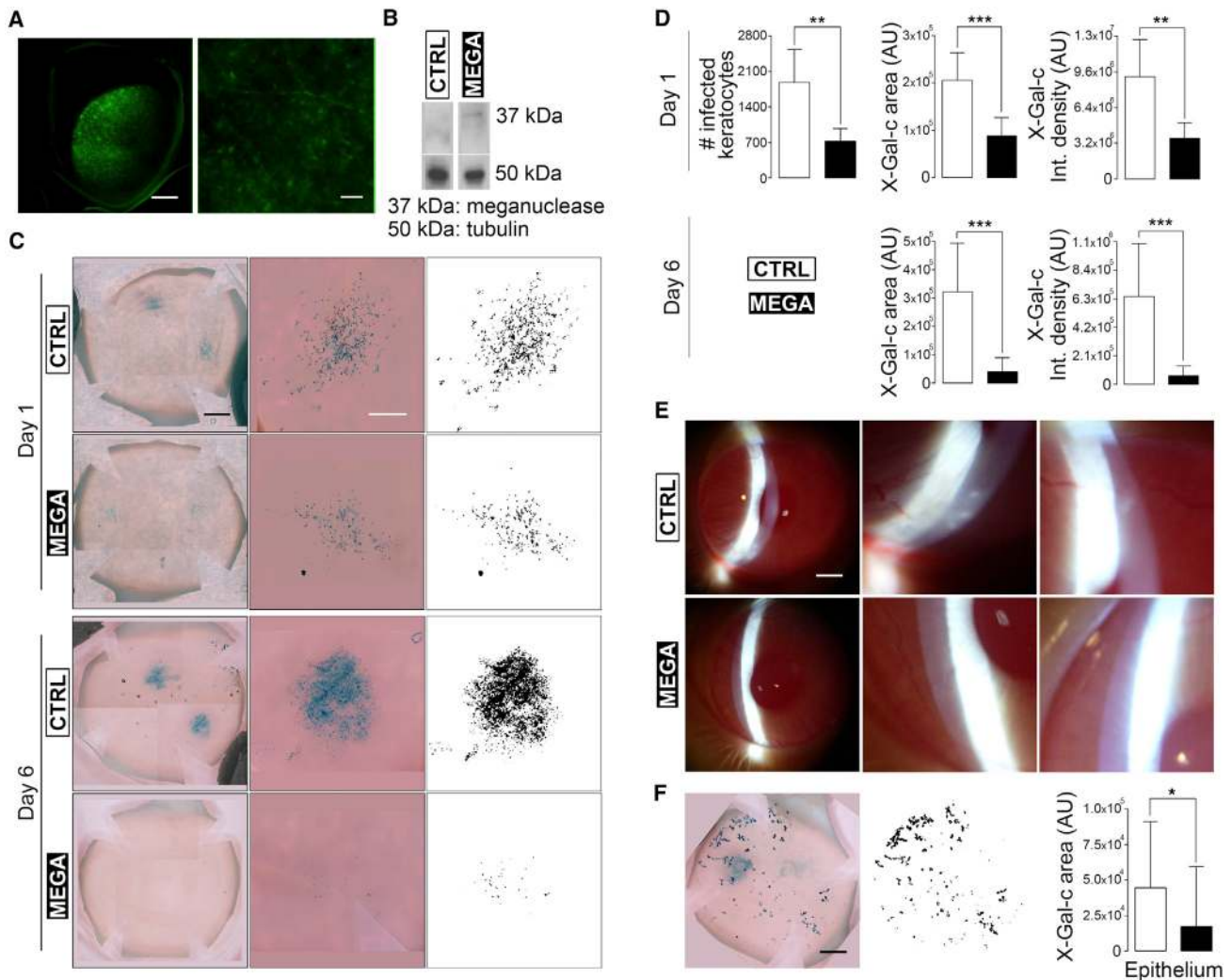


Figure 3. Intrastromal injection of the meganuclease-coding vector into *in vivo* rabbit corneas inhibited rHSV-1 corneal infection

(A) Enhanced green fluorescent protein (EGFP) expression following intrastromal injection of adeno-associated virus (AAV)-EGFP. Reporter direct fluorescence was observed with a macroscope 2 weeks later (scale bars, 2 mm [left] and 100 μ m [right]). Representative view (Z-stack projection, two experiments, eight eyes). (B) AAV-meganuclease (MEGA) induced MEGA expression. Western blot analysis of the stroma 2 weeks after intrastromal injection (one experiment, $n = 4$). (C, D) Observation and quantification of stromal X-Gal-generated chromophore (X-Gal-c) release 1 and 6 days postinjection of 25 μ L of rHSV-1 solution (2,000 PFUs). Macroscopic examination (C, left panel; scale bar, 2 mm), Z-stack maximum intensity projection of stromal X-Gal-c release (C, central panel; day 1, three experiments with $n = 4$ eyes; 6 days, two experiments with $n = 6$ eyes; scale bar, 1 mm), and ImageJ-treated outlines of X-Gal-c in the former (C, right panel). (D) X-Gal-c-colored keratocyte counts, labeled areas and integrated densities on days 1 and 6. AAV-CTRL and AAV-MEGA results are expressed as mean $\pm$ SD (day 1, $n = 8$ vector injection sites; day 6, $n = 12$ sites). Two-sided Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (E) Slit-lamp examinations of the corneas 6 days postinfection. Corneal infiltrates seen in the AAV-CTRL-treated eyes as hazy grayish areas, are not detected in the AAV-MEGA-treated corneas. Narrow slit-beam image (left panel, scale bar, 2 mm) and magnified images (middle and right panels). (F) Epithelial infection 6 days after stromal rHSV-1 injection. Quantification of epithelial X-Gal-c. Macroscopic observation (left panel; scale bar, 2 mm), ImageJ outlines of epithelial X-Gal-c (central panel), and X-Gal-c area analysis (right panel; one representative experiment, $n = 12$). AU: arbitrary units.

~61% lower in the AAV-MEGA-treated group, compared with AAV-CTRL (Mann-Whitney highest $p = 0.03$).

Because stromal herpetic infection is known to spread to the fifth cranial nerve ganglion through retrograde transport,^{17,18} it was tempting to hypothesize that corneal meganuclease treatment might also prevent viral infection of the brainstem. qPCR detection of rHSV-1

DNA in rabbit periobex 6 days after corneal stroma inoculation was less frequent in the AAV-MEGA pretreated group (qPCR, cycle threshold ≤ 35 in six out of nine AAV-CTRL-group samples compared with one out of nine in the AAV-MEGA group). Hence, AAV-MEGA pretreatment of corneal stroma seems to protect against HSV infection spreading not only to the epithelium but also to the trigeminal ganglion.

DISCUSSION

Despite advances in surgical techniques and antiviral therapies, HSK-caused losses of corneal transparency and vision are rarely treated by corneal transplantation because of the poor prognosis of keratoplasties in this context.¹⁹ Endothelial keratoplasty, a recently developed surgical technique, enabling selective replacement of the corneal endothelial layer, is considered mini-invasive surgery resulting in less surgical trauma and is associated with a lower rejection rate. Although this technique improved prognoses, the prognosis remains poor after HSK.³ Herein, we described a novel gene-therapy system using meganuclease, which successfully inhibited HSV-1 infection in *in situ* corneal stroma and corneal transplants *in vivo*.

The cornea is a gene-therapy target-of-choice in light of its easy access for vector delivery, avascularity, and relatively privileged immune status.²⁰ Moreover, corneal transparency and location in the front of the eye allows easy assessment of the clinical evolution posttreatment.

Different vectors were assessed for gene delivery as human gene therapy. AAV-based gene therapies have been assessed in clinical trials to target diseases affecting retina, muscles, blood, and immune cells (clinicaltrials.gov, September 2021). Nevertheless, no ongoing clinical trial in humans is assessing gene therapy in the cornea. rAAV vectors seem to have an acceptable safety profile and gene therapy using them has the advantage of their nonintegrative capacity, thereby limiting the mutagenesis risk.^{9,21} Furthermore, the limited replicative properties of corneal endothelial cells and keratocytes should achieve a theoretical long-lasting effect of the delivered gene and do not require iterative treatments.

Gene-editing systems include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), CRISPR-Cas9, and meganucleases. The meganuclease family has received relatively less attention in recent years than other gene-editing systems, such as CRISPR-Cas9. While production and design of meganucleases are more delicate undertakings, their relatively compact size allows easy packaging. In addition, the large protein-DNA-contact area, with the same protein domain triggering DNA binding and cleavage, suggests that meganucleases might have superior specificity for their targets and fewer off-target events.²² However, rapid advancements made with the other endonuclease classes, particularly the CRISPR-Cas system, might favor them for future work.

CRISPR-Cas9 was recently used *in vitro* to inhibit HSV replication²³ by targeting several essential HSV proteins. Wayengera²⁴ developed a ZFN targeting HSV-2, but possible off-target activity and induced genomic mutations represent some of the concerns regarding CRISPR-Cas9 and ZFNs. Further studies comparing efficiencies of meganuclease therapy with a CRISPR-Cas9-editing system are warranted. Nonviral-delivery systems could also be considered (nanoparticles, vesicle, polymer, etc.).

A murine model of herpes stromal keratitis reactivation recently demonstrated the efficacy of a CRISPR-Cas9 system targeting UL8/UL29 and showed a low level of off-target effects.²⁵

Recently, Aubert et al.¹⁸ reported *in vivo* HSV-1 targeting using a meganuclease-AAV vector. The authors demonstrated the inhibition of active HSV replication in murine trigeminal ganglion neurons. Using next-generation sequencing technology, they demonstrated low-level mutation detection in the off-target sequences of DNA samples from treated animals and mutation frequencies and distributions were similar across the sites to the levels observed in control animals. In addition, Elbadawy et al.²⁶ also demonstrated the feasibility of using a meganuclease-AAV vector to protect *ex vivo* human corneal endothelium against HSV-1 infection, suggesting the interest and feasibility to pretreat human corneas in eye banks using meganuclease-encoding vectors prior to grafting patients affected by HSK.

Our study demonstrated the possibility to genetically modify corneas *ex vivo* in order to protect subsequent grafts from HSV endothelial infections. While this method does not reproduce the natural history of herpetic recurrences, the high titers of HSV used in our system represent a strong proof of efficacy. In addition, *ex vivo* transfection of corneal grafts represents a safer procedure limiting the risk of extra-ocular transduction reducing the potential risk of mutagenesis and allowing quality controls of corneal tissues prior to grafting.

Conclusion

Previous conventional anti-herpes treatments also limit virus proliferation. However, for the more severe lesions, like recurrent stromal keratitis or following keratoplasty, lifelong treatment is required but patients rarely adhere to it. Herein, we showed that gene therapy in both of those clinical situations could represent a promising strategy to protect the cornea against opacification and protect corneal transplants.

MATERIALS AND METHODS

Animals, organs, and cells

Full-sister New Zealand White rabbits (25–28 weeks old; Hypharm, Roussay, France) were used for corneal transplantation; other experiments involved outbred rabbits (13–14 weeks old). Procedures were done under pharmacological restraint using intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg), and topical anesthetic (oxybuprocaine, Théa), except keratoplasties that were performed under general anesthesia. Animal care and treatment in this investigation were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were performed under veterinary supervision at the Institute for Clinical Animal Research (IRCA, Maison Alfort, France) after ethical comity approval. The Eye Bank of Normandy (Rouen, France) provided human corneal buttons unsuitable for transplantation. Telomerase-immortalized corneal fibroblasts of human origin (hepatoma-transmembrane kinase [HTK] cell line) were kindly provided by J. Jester (Avin Herbert Eye Institute, University of California, Irvine, CA). Organs and cell lines were maintained at 37°C under 5% CO₂ atmosphere in DMEM Low Glucose (Life Technologies #21885) supplemented with 10% fetal calf serum (FCS; Life Technologies) and antibiotic-antimycotic cocktail (ABAM; Life Technologies #15240). COS-7 cells (ATCC CRL-1651) were cultured under the same conditions except DMEM High Glucose (Life Technologies #119652) was used.

Table 1. Primers used

Sense	Sequence
UL18 virus gene	
Forward	ATCTGCAGGAGAGCGTTACGTA
Reverse	AGGGAACCAACATCATCCTTACGCT
Rabbit β -actin gene	
Forward	GGTCAACGCTGTCATCTTGC
Reverse	TGAATCAGAGCCCTTGCTCG
Rabbit myogenin gene	
Forward	CTTGCAGACCATATGCTTGCAG
Reverse	CTCAGTTGGGCATGGTTTCATC
HSV-1m2 messenger	
Forward	TCTACCCAACTGCAACCTTCT
Reverse	ATTCAAGGCAGCCACCTGATCCA
Rabbit glyceraldehyde-3-phosphate dehydrogenase messenger	
Forward	AGAAGGCCGGGGCTCATTGAA
Reverse	AGTTGGTGGTGCAGGATGCGTT
Human Cpxm1	
Forward	TTACAAGGCCATGAGGAAGG
Reverse	ATACAAGCGCAGTCACAGCA

Viral vector selection

The following EGFP reporter-conjugated viral vectors were used to assess endothelial transduction: rAAV-2/5 (recombinant AAV-2 genome with an expression cassette driven by human enhanced-cytomegalovirus [ECMV] promoter and packaged in an AAV-5 capsid; Gene Therapy Laboratory, INSERM UMR1089, Nantes, France); rAAV-2/1 (single-stranded vector pseudotyped with a serotype-1 capsid allowing transgene expression from the ECMV promoter; Vectalys).

Meganuclease, viral vectors, and HSV-1 strains

Meganuclease engineering methods^{27,28} used and *in vitro* testing⁷ have been described before. The meganuclease is an I-CreI variants characterized in that at least one of the two I-CreI monomers has at least two substitutions, one in each of the two functional subdomains of the LAGLIDADG core domain, so that the variant is able to cleave a DNA target sequence from the herpes simplex virus (HSV) genome. After selecting a viral vector adequate for corneal endothelium transduction (Figure S1), experiments involved single-stranded AAVs based on the AAV-2 genome, equipped with an expression cassette of an intervening sequence (produced as previously described¹⁸): (1) coding for the EGFP [AAV-EGFP]; (2) coding for a meganuclease directed against either the *ICP0* gene (HSV-1m4) or the capsid protein *VP5* gene encoding UL19 (HSV-1m2; AAV-MEGA)⁷; (3) noncoding EGFP sequence in antisense orientation, with three stop codons close to the promoter (AAV-CTRL), driven by the human ECMV promoter, and packaged in an AAV-1 capsid (vectors AAV-2/1) (Vectalys). The HSV-1m2 (AAV-MEGA) targets UL19 gene encoding the HSV-1 major capsid protein (targeted sequence: ATAACTCACACACGGCGTCTCTGG).

Unless otherwise stated, herpetic infection experiments implicated the recombinant strain F (rHSV-1),⁷ containing two LacZ-expression cassettes. rHSV-1 was purified after two-cycle amplification in COS-7 cells infected at 0.01 multiplicity-of-infection (MOI) during a 52-h incubation and 100X concentration ($80,000 \times g/1 \text{ h}/4^\circ\text{C}$ and resuspension in balanced salt solution [BSS] [Beaver-Visitec #585831]). Plaques were titrated through 2 days-infection of COS-7 cells under agar²⁹ (titers of 1×10^7 or 3×10^8 PFU/mL). The wild-type strain SC16³⁰ was cultured in baby hamster kidney (BHK) cells, concentrated as above and titered in Vero cells (1.6×10^8 PFU/mL).

Western blot and qPCR

Meganuclease expression was tested using a primary antibody against I-CreI, endonuclease isolated from *Chlamydomonas reinhardtii*, as previously described.⁷ Cells were lysed using RIPA buffer (Sigma #R0278, Protease Inhibitor Cocktail Sigma #P8340 and phenylmethylsulfonyl fluoride Sigma #P7626). Proteins (20 μg) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were incubated with a primary rabbit polyclonal antibody against I-CreI (diluted 1:20,000) followed by a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (diluted 1:5,000). Specific protein bands were visualized with a chemiluminescence reagent (Santa Cruz Biotechnology). A rabbit antibody directed against β -tubulin (Cell Signaling Technology) was used as the loading control.

RNA was isolated using an RNeasy kit (Qiagen #74134) and subjected to on-column DNase digestion (Qiagen #2500120), according to the manufacturer's instructions. cDNA was synthesized using SuperScript III First-Strand Synthesis System (Life Technologies #18080-51), according to the manufacturer's instructions. qPCR comprised 40-cycle amplification using gene-specific primers (Table 1) and a Power SYBR Green PCR Master Mix (Life Technologies #4367659) on a 7500 Real-Time qPCR System (Applied Biosystems). qPCR parameters were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Triplicate measurements were obtained and normalized to reference-gene levels. For qPCR rHSV-1 genome quantification in aqueous humor, the result was normalized to the reaction volume. Amplicons were quantified using the standard-curve method.

AAV-2/1 transduction capacity in HTK cell line

HTK cells (4×10^5 per well, six-well plate) were seeded and transfected 16 h later with 32.5×10^{10} AAV-GFP, AAV-MEGA, or AAV-CTRL vg in ABAM-free medium. The vector was discarded 24 h later. Direct fluorescence was observed with Nikon Instruments AZ100 Multizoom (macroscope). Seventy-two hours after vector introduction, cells were fixed in 2% paraformaldehyde (PFA) and nuclei counterstained with DAPI. Total cell proteins were extracted for western blot analysis.

Antiviral potential of AAV-MEGA in HTK cells

HTK cells (1×10^5 per well, 24-well plates) were treated with 17×10^{10} AAV-MEGA or AAV-CTRL vg for 48 h in ABAM-free medium. Then cells were incubated for 1 h at room temperature with serial rHSV-1 dilutions to reach 0.5, 0.05, or 0.005 MOIs and further

cultured in ABAM-free medium. After rinsing with Dulbecco's-phosphate-buffered saline (D-PBS), cultures were continued in ABAM-free medium for 24 h before processing for analysis. (1) A β -Galactosidase Enzyme Assay System (Promega kit #E2000; hydrolysis of its colorless substrate ortho-nitrophenyl- β -galactoside, to *o*-nitrophenol, a yellow chromophore) was used on total cell protein extract, according to the manufacturer's instructions, before TECAN-Infinite M1000 measurement of chromophore absorbance. Total protein concentration was estimated by Lowry's assay (Bio-Rad #500-0112). (2) After cell lysis (50 mM Tris-HCl pH8, 200 mM NaCl, 5 mM EDTA, 0.5% SDS), DNA was isolated using DNA Isolation Kit (Roche Diagnostics #03115 879,001), according to the manufacturer's instructions. qPCR enabled measurement of UL18 VP5, human β -actin, and carboxypeptidase X1 (*Cpxm1*) gene expressions.

HSV in vivo stromal keratitis model

Rabbits received peribulbar triamcinolone acetate (250 μ L Kenacort-retard, Bristol-Myers Squibb) and intrastromal AAV-2/1 injection the next day via an insulin syringe (Terumo Myjector, 0.5 mL) and a 29-G needle inserted 2 mm from the limbus caused temporary edema, whose extent depended on the volume injected. Rifamycin cream (Chibret 1,000,000 IU/100 g, Théa) was then applied into the conjunctival sac. To evaluate AAV-2/1 transduction capacity, 150–200 μ L of a vector-containing solution (2×10^9 vg/ μ L in BSS) was injected into the corneal stroma. Corneas were excised 2 weeks later. Direct fluorescence of whole-mount corneas exposed to AAV-EGFP was analyzed under the macroscope after fixation in 4% PFA. Soluble proteins extracted from corneas exposed to AAV-MEGA or AAV-CTRL (after deepithelialization) with T-PER (Life Technologies #78510)³¹ were subjected to western blot analysis.

To assess AAV-MEGA efficiency, 75 μ L of AAV-MEGA or AAV-CTRL vector solution (2×10^{11} vg) were injected into the stroma at two diametrically opposed sites. Two weeks later, after pupil dilation (topical mydriatic, Théa, and phenylephrine, Europhtha), the injection sites were inoculated with rHSV-1 (2×10^3 PFU in 25 μ L of BSS), in combination with fluorescein (500 $\times$ dilution of Fluorescein Faure 0.5%, Théa), using a precision syringe (Hamilton Gastight, 50 μ L, 30-G removable needle [RN]).

Keratitis analysis

Corneas were photographed daily until day 6 via slit-lamp biomicroscopy before and after fluorescein instillation. Corneas were then excised, fixed in 4% PFA, permeabilized with 0.02% Tergitol (Sigma #NP40S) and 0.01% sodium deoxycholate (Sigma #30970), labeled for 16 h at room temperature in 0.1% X-Gal solution, DAPI-counterstained, and whole mounted for macroscopy examination. X-Gal-generated chromophore (X-Gal-c) release in the stroma was studied using 40- μ m-interspaced Z-stack images taken at $\times 8$ magnification. The epithelial and stromal chromophore coloration were identified by their positions in the Z stack. X-Gal-generated chromophore release was assessed for inoculated center and peripheral zones. Stromal X-Gal-generated chromophore release was quantified on maximum intensity Z-stack projections after epithelial signal

subtraction. Yellow keratocytes were counted manually. Epithelial X-Gal-generated chromophore was quantified after subtracting stromal chromophore expression, and normalization to corneal area. Image analysis used ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA, <https://imagej.nih.gov/ij/>).

rHSV-1 detection in trigeminal ganglia

Brain removal followed the protocol described by Tyson.³² The brain stem was sectioned at the level of the ventral groove, demarcating the pons varolii and the medulla oblongata, and cut caudally 1 cm apart. DNA was extracted after protein digestion (Proteinase K, 0.5 mg/mL) and phenol-chloroform extraction. qPCR quantified UL18 VP5, rabbit β -actin, and myogenin gene expressions, as described above.

Ex vivo corneal endothelium transduction

Excised rabbit corneas were stored endothelial side up in preservation medium (Cornea Max, Eurobio) for 3 days. Corneas were incubated in DMEM containing AAV-EGFP vectors for 4 h before adding FCS and ABAM. Following a 3-day incubation, culture medium was replaced twice a week until final processing. Specimens were stained with a cell tracker (5 μ M CellTracker Orange CMRA, Life Technologies #C34551), fixed in 0.5% PFA and counterstained with DAPI. To estimate transduction efficiency, the endothelial surface was examined up to 39 days postinoculation under the macroscope or a confocal microscope Leica TCS SP2. For AAV-MEGA and AAV-CTRL vectors, corneas were exposed to 2×10^{11} vg, as described above. Two weeks later, the endothelium was then scraped off the cornea for total protein extraction and further western blot analysis.

Meganuclease efficiency in an organ-culture model of corneal endothelial HSV infection

Corneas cultured for 2 weeks were transferred in culture medium containing SC16 or rHSV-1 (five corneas for each condition). Excess virus particles were discarded 3 h later and corneas were either incubated in DMEM supplemented with FCS and DEAE-dextran hydrochloride (5 μ g/mL, Sigma #D9885) for infection in liquid medium, or cultured in a 3:1 mix of DMEM DEAE-dextran (5 μ g/mL), supplemented with an aqueous solution of Lonza SeaKem ME agar (final concentration: 0.6%) for infection under agar. Corneas were fixed in 0.5% PFA and processed 40 h later for CMRA fluorescence, X-Gal-chromophore release or immunolabeled with antibodies targeting the HSV-1 envelope glycoprotein D (gD) and counterstained with DAPI. The anti-HSV-1 labeling included permeabilization with 0.05% Triton X-100, incubation with the primary antibody (goat antibody HSV-1 gD [vN-20], Santa Cruz Biotechnology #SC-17540; diluted 1:100 in D-PBS containing 0.01% Triton X-100 and 1% BSA) and then with the secondary antibody (donkey anti-goat IgG [H + L] Alexa Fluor 488 conjugate, ThermoFisher Scientific #A-11055; diluted 1:1000). Endothelial surfaces were examined with a macroscope.

Endothelial X-Gal-generated chromophore analysis in organ-cultured corneas

Corneas transfected with AAV-MEGA or AAV-CTRL were infected with recombinant (300 rHSV-1 PFU) or wild-type strain (300 or 600

SC16 PFU) under agar for 40 h and further processed for X-Gal chromophore, as described above. The X-Gal-generated chromophore signal was assessed on macroscopic views of the endothelial surface and further processed (assembly, maximum intensity projections of Z-stacks). Endothelial plaques, defined as discoidal collections of cells containing X-Gal-generated chromophore, were counted and normalized to the endothelial area measured with ImageJ. Cells containing X-Gal-generated chromophore were counted for 50 plaques or, by default, for all plaques. SC16-infected corneas were analyzed on macroscopic views of CMRA fluorescence and gD immunolabeling. Because we had observed that the CMRA signal was condensed in infected cells (Figure S1), the endothelium was considered to be infected only where both markers colocalized and, thus, avoided misinterpretation related to nonspecific antibody binding. Plaques were counted on the whole corneal surface and their outlines traced with ImageJ to estimate the area of the infected endothelial zone.

Tests on human corneas

Using the technique described for rabbit, human cornea pairs were exposed to AAV-CTRL or AAV-MEGA (5×10^{11} vg) for 3 h then cultured for 12 days in vector-free medium before inoculation with 300 or 20×300 rHSV-1 PFU for 2 h and further cultured under agar for 40 h. Infection levels were then assessed as described above.

Assays in the *in vivo* rabbit model of induced herpetic endotheliitis

Rabbits received a 100- μ L intravitreal injection of triamcinolone acetonide, followed the next day by 100 μ L of a vector solution containing 3.5×10^{11} AAV-EGFP, AAV-MEGA, or AAV-CTRL vg injected intracamerally (Gauge 30S, RN-Hamilton on 800 RN/250 μ L) through a 1.2-mm self-sealing tunnel incision made with a stab knife in the cornea, 1.5 mm behind the limbus. Then antibiotic ointment was applied. Reporter direct fluorescence and DAPI counterstaining were studied at the endothelial surface 14 or 39 days after AAV-EGFP exposure and corneal excision, as described above. For corneas exposed to AAV-MEGA or AAV-CTRL, endothelium was scraped off the corneas 14 days later for RNA extraction. After reverse transcription, HSV-1m2 and rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were quantified by PCR. Meganuclease expression was verified by western blot.

Intracameral injection of rHSV-1

Fourteen days posttransfection, as described above, the aqueous humor was replaced by 300 μ L of Corneamax (Eurobio) plus 8% dextran and, 2 min later, 100 μ L of the same medium containing 3×10^4 rHSV-1 PFU was injected (13 control and experimental samples in four experiments for 40-h infections, and six eyes for each group in two experiments for a 64-h infections; 8×10^4 rHSV-1 PFU for nine controls and eight experimental animals in three experiments for 40-h infections with fluorescein included in the inoculum to control the injected volume). Slit-lamp eye examination, aqueous humor sampling, and corneal button removal were done 40 or 64 h postinoculation. Corneas were processed for X-Gal-generated chromophore

release, and counterstained with DAPI and microscope examination, as described above.

Analysis of macroscopic photos

Endothelial plaque subtype was determined by comparing X-Gal-generated chromophore and DAPI signals. Noneroded plaques were defined as endothelial zones where X-Gal-generated chromophore in cells was clustered into discoidal islets with apparently normal cell density, compared with noninfected tissue (based on DAPI signals). An eroded plaque was a portion of the endothelium containing at least one chromophore-colored discoidal shaped cell with a nucleus-free area (identified on DAPI-stained images).

Inflammatory cellular deposits, called keratic precipitates (KPs), could be seen on plaques on chromophore-colored images, appearing as whitish or translucent masses corresponding to collections of nuclei smaller than endothelial cell nuclei on DAPI-stained photos. These plaque types were identified and counted manually for the entire endothelial surface after image assembly with Adobe Photoshop CS4. The X-Gal-generated chromophore signal of plaques was outlined with ImageJ. The resulting outlines were superimposed and aligned on the DAPI-stained image. For eroded plaques, the outlines were completed by delineating the areas devoid of DAPI signal. The encircled areas, which correspond to the infected endothelial zone, were finally measured with ImageJ and normalized to the number of examined plaques.

Slit-lamp examination

The clinical inflammatory response was examined at the level of the anterior chamber by referring to the standardized grading system of a uveitis flare,³³ based on the assessment of the iris haziness and lens details graded as follows: clear chamber (0), faint (1+), moderate (2+), marked (3+), or intense (4+). An intense flare (4+) corresponds to fibrin presence. KP presence or absence was graded 1 or 0, respectively, in five corneal sectors (central, superior, inferior, temporal, nasal). The summed grade of the five sectors for each cornea was calculated to reflect the KP extent. Focal edema in the overlying stroma was evaluated like KPs.

rHSV-1 detection in aqueous humor

In the second series of experiments ($n = 8$), 20 μ L of aqueous humor were added to 80 μ L of 0.1% Triton X-100 and incubated for 5 min at 98°C. The samples were then spun down, diluted twice, and processed for qPCR to quantify UL18 VP5 gene expression, as described above. Because the aqueous humor is devoid of cells under normal physiological conditions, quantifications could not be normalized; in the case of inflammation, its cell content depends on the inflammatory activity in the anterior chamber.³⁴

Corneal transplant preparation

Corneas excised from adult rabbits were incubated for 24 h at 37°C endothelial side up in 3.8 mL of Corneamax containing 3.55×10^{11} AAV-MEGA or AAV-CTRL vg. Samples and media were then transferred to a vial containing 21 mL of Corneamax, and incubated for 30 h. Medium was replaced with 25 mL of deswelling medium

(Corneajet, Eurobio) and maintained at 37°C for 12 h. Corneal buttons not destined for keratoplasty were further cultured for 11 days before qPCR analysis of the meganuclease expression level in the endothelium.

Penetrating keratoplasty

Keratoplasties were performed after pupil dilation under general anesthesia induced by intramuscular injection of medetomidine (200 µg/kg), ketamine (20 mg/kg), and diazepam (0.5 mg/kg) and maintained with intravenous ketamine (1 mg/kg) combined with topical anesthetic (tetracaine 0.5% eye drops; Faure-Novartis), and anti-inflammatory analgesics (1 mg/kg subcutaneous Metacam; Boehringer). Host or donor corneas from full sisters were trephined with an 8.0- or 8.5-mm biopsy punch, respectively. After trephination, the anterior chamber was filled with viscoelastic solution (1% sodium hyaluronate ophthalmic viscosurgical devices; Beaver). The transplant was held in place with four interrupted sutures and secured with a running suture (9-0 nylon). Viscoelastic solution was replaced by BSS before tightening of sutures. Tarsorrhaphy (5-0 silk) closed two-thirds of the palpebral fissure. Oxytetracycline-dexamethasone ophthalmic ointment (Sterdex, Théa) was applied. During the 6 days postsurgery, Sterdex ointment was applied twice a day, animals received subcutaneous injections of Metacam (1 mg/kg), and enrofloxacin (10 mg/kg Bayer) was added to the drinking water. The next week, treatment consisted of applying Sterdex and vitamin A ointment (Dulcis, Allergan) twice a day. This regimen was renewed for 1 week after tarsorrhaphy removal, when the transplant showed complete reepithelialization, then Sterdex was applied twice a day. On day-28 postgrafting, corneal sutures were removed and 250 µL of triamcinolone acetonide were injected via the peribulbar route. Vitamin A ointment was applied twice daily until rHSV-1 inoculation. On day 42, 100 µL of triamcinolone acetonide was injected intravitreally, and 24 h later 9×10^4 rHSV-1 PFU diluted in Corneamax and 8% dextran was inoculated into the anterior chamber through a self-sealing tunnel incision made in the recipient cornea. In three of the five experiments, fluorescein was added to assess the actual inoculum volume. Forty hours later, the cornea was examined, and aqueous humor and corneal buttons were collected for analysis, as described above.

Statistical analyses

Analyses were computed with XLSTAT-Pro (Addinsoft, New York, NY): two-sided Mann-Whitney test; Spearman's rank correlation (R_s); Fisher's exact test; two-sample Kolmogorov-Smirnov test; $p \leq 0.05$ defined as statistically significant.

DATA AVAILABILITY STATEMENT

All data associated with this study are reported in the paper or the Supplementary Materials.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2022.11.006>.

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AUTHOR CONTRIBUTIONS

Conceptualization: E.E.G., B.C., J.A.S., M.L.; Methodology: B.C., E.E.G., M.L.; Investigation: B.C., E.E.G., D.P.; Resources: R.G. provided the meganuclease; Visualization: B.C., E.E.G., D.G., S.M.; Funding acquisition: E.E.G., J.A.S., R.G., M.L.; Project administration: B.C., E.E.G.; Supervision: E.E.G.; Writing – original draft: B.C., E.E.G., D.G., S.M.; Writing – review & editing: E.E.G., D.G., S.M.

DECLARATION OF INTERESTS

R.G. works for Collectis, which produced the meganuclease.

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