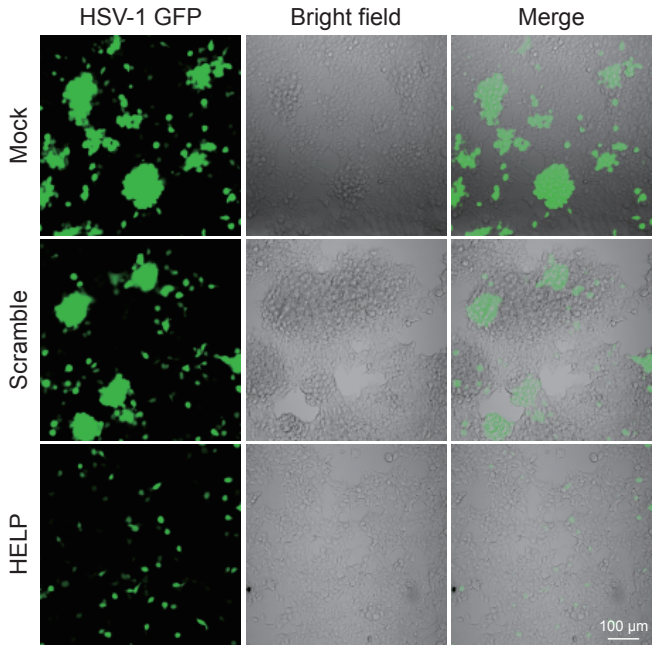
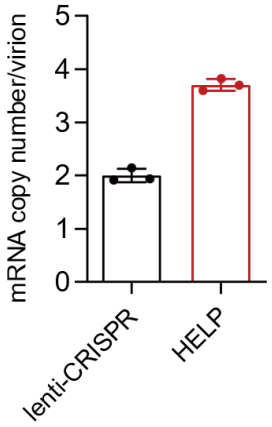
Supplementary Information

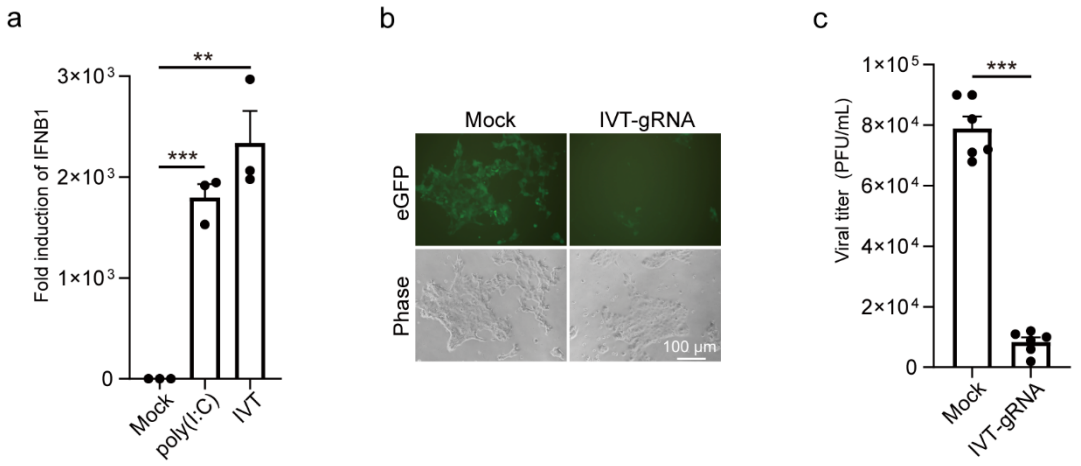
Targeting herpes simplex virus with CRISPR-Cas9 cures herpetic stromal keratitis in mice



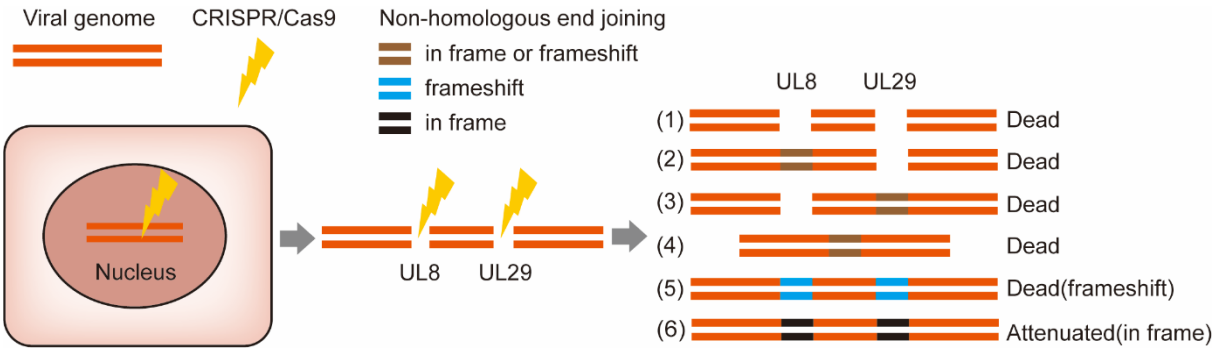
Supplementary Fig. 1 The antiviral activity of HELP in 293T cells. Cells were seeded 24 h before transduction of HELP (400 ng p24) at a density of 4×10^4/well. 24 h after transduction, cells were infected with HSV1-GFP. Photographing was performed two days after HSV1-GFP infection (MOI=1). Scale bars, 100 μm. The experiment was repeated twice with similar results.



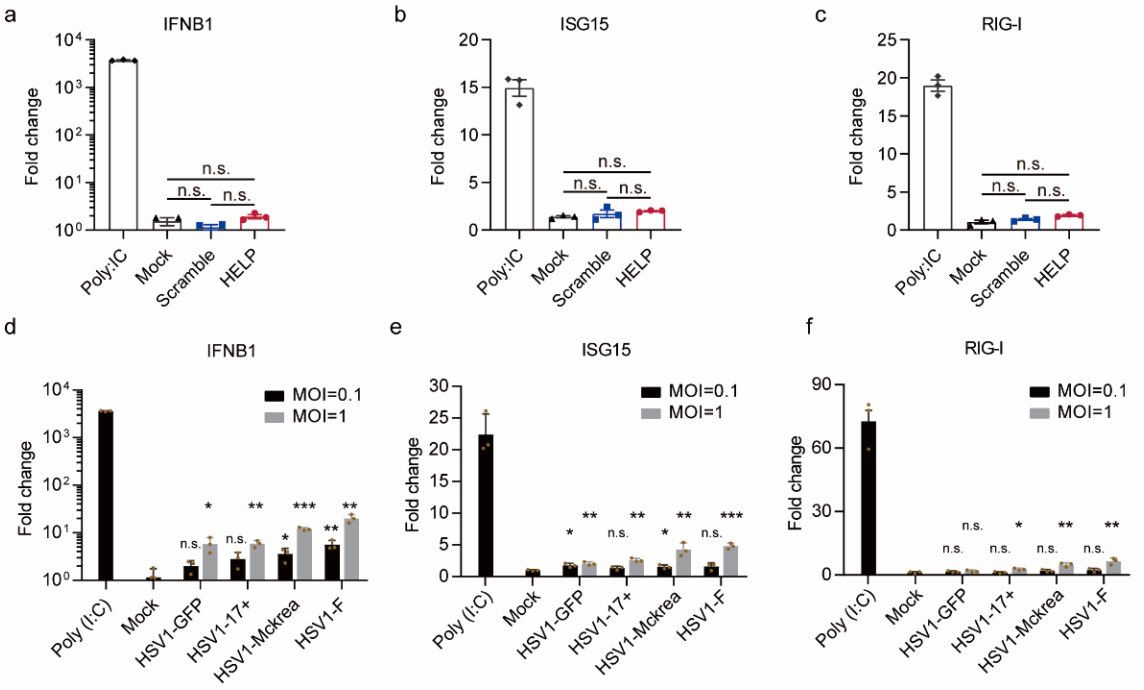
Supplementary Fig. 2 The copy number of Cas9 mRNA in each HELP particle. Lenti-CRISPR is a standard lentiviral vector encoding Cas9 and gRNA. To detect the copy number of Cas9 mRNA in each HELP particle, the same amount p24 of HELP and lenti-CRISPR was used to extract total RNA and synthesized to cDNA for RT-qPCR. Data of HELP was normalized to lenti-CRISPR. n=three biologically independent replicates. Data and error bars represent mean ± SEM.



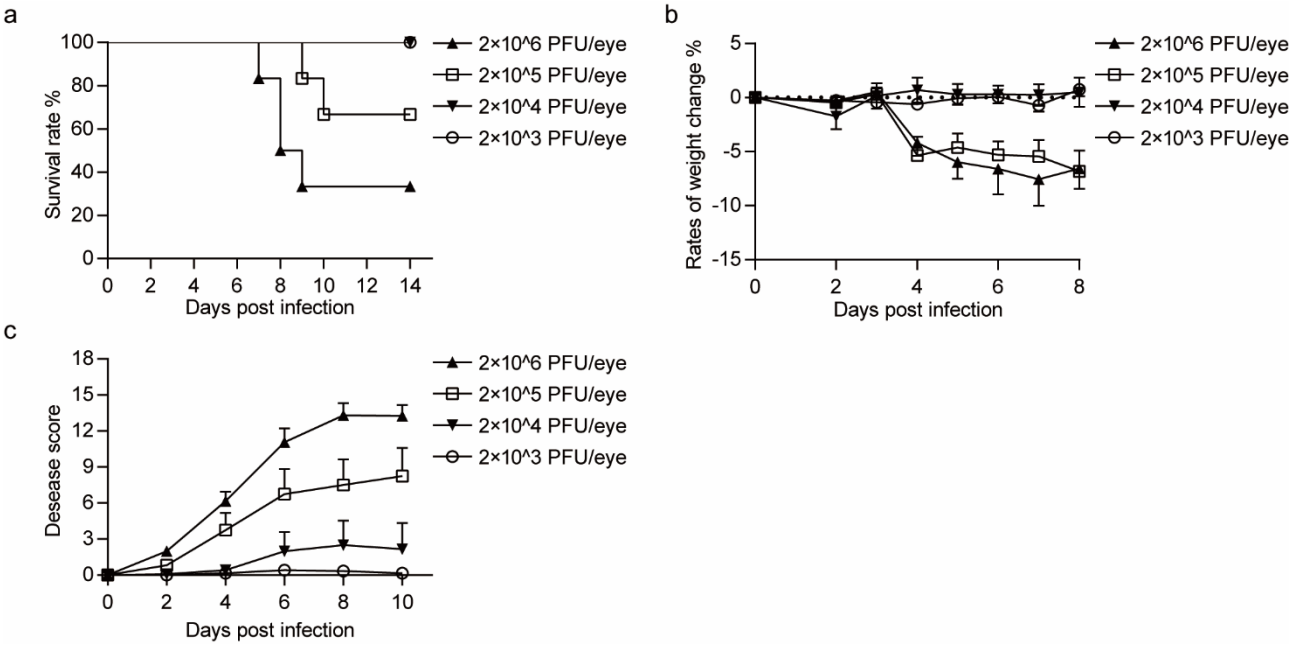
Supplementary Fig. 3 Innate immune stimulator inhibits HSV1-GFP replication. a, Fold induction of IFNB1 by UL8-targeting *in vitro* transcribed gRNA (IVT-gRNA) and poly(I:C) in HaCaT cells. RNA was isolated from HaCaT cells 6 h after infection or transfection. IVT-gRNA vs. Mock, Mock vs. poly(I:C), P=0.0002; Mock vs. IVT-gRNA, P=0.0018. n=three biologically independent replicates. **b**, The antiviral activity of IVT-gRNA. Representative fluorescent and phase-contrast photographs of HaCaT cells 24 h after IVT-gRNA transfection. IVT-gRNA transfection was performed 1 h after HSV1-GFP infection (MOI=1.5). No Cas9 was provided. **c**, Plaque assay analysis of infectious viruses in supernatants harvested from mock and IVT-gRNA treated cells (from **b**). P< 0.0001. n=six biologically independent replicates. Data and error bars represent mean ± SEM. Unpaired two-tailed Student's t-tests were performed. Scale bars, 100 μm.



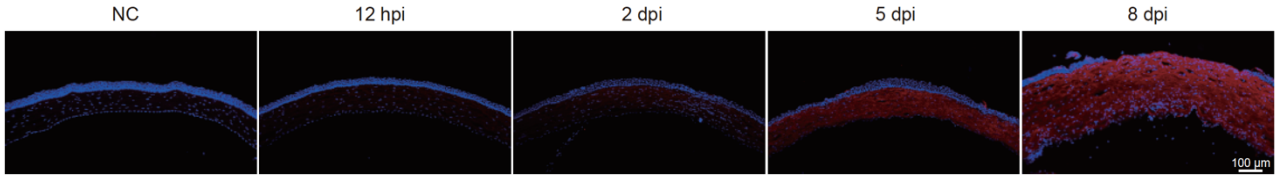
Supplementary Fig. 4 Illustration of the possible outcomes of HSV-1 genome after HELP cleavage. If the DSBs are not repaired (outcome 1-3), the viral genome un-replicable. If the breaks are repaired but causing large deletion or frameshift, the virus is also dead (outcome 4 and 5). If the DSBs are repaired and in frame, the virus is alive but attenuated (outcome 6).



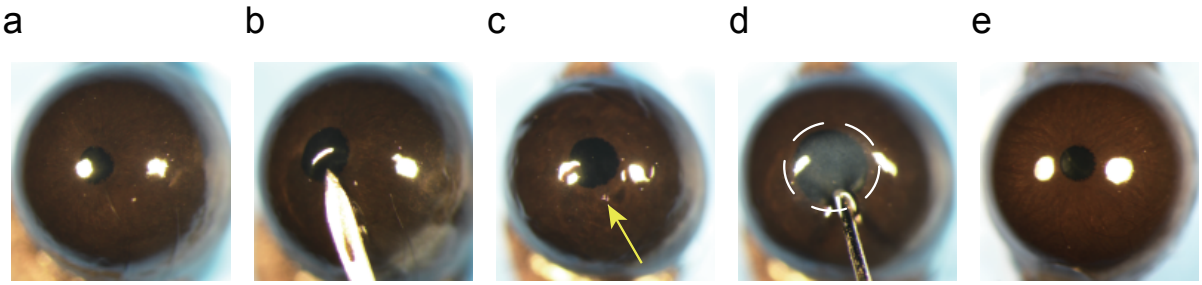
Supplementary Fig. 5 The innate immune response induced by HELP and different HSV-1 strains in THP-1 derived macrophages. Cells were harvested for IFNB1, ISG15 and RIG-I analysis by RT-qPCR 6 h after transduction (**a-c**) or infection (**d-f**). **d**, Mock vs. HSV1-Mckrea and HSV1-F at MOI=0.1, *P*=0.0293 and 0.0059; Mock vs. HSV1-GFP, HSV1-17+ and HSV1-F at MOI=1, *P*=0.0198, 0.0019 and 0.0015, respectively. Mock vs. HSV1-Mckrea at MOI=1, *P*<0.0001. **e**, Mock vs. HSV1-17+, HSV1-Mckrea and HSV1-F at MOI=1, *P*=0.0162, 0.0036 and 0.0042, respectively. **f**, Mock vs. HSV1-GFP and HSV1-Mckrea at MOI=0.1, *P*=0.0146 and 0.0387; Mock vs. HSV1-GFP, HSV1-17+, HSV1-Mckrea and HSV1-F at MOI=1, *P*=0.0034, 0.0010, 0.0058 and 0.0001, respectively. n=three biologically independent replicates. Data and error bars represent mean ± SEM. One-way ANOVA with Dunnett's multiple comparisons test (**a-c**) and unpaired two-tailed Student's t-tests (**d-f**) were performed. n.s.=non-significant.



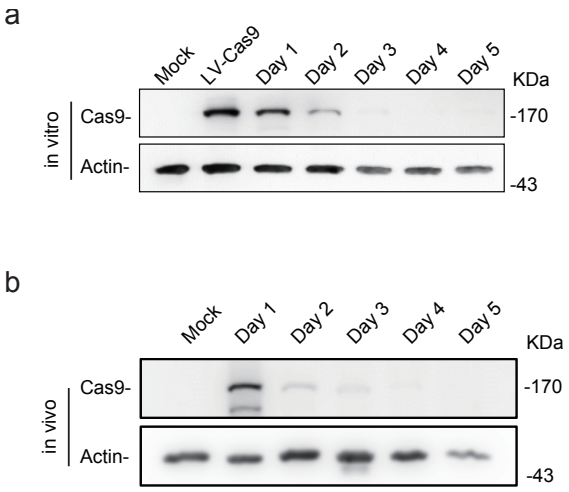
Supplementary Fig. 6 Dose-response and keratitis symptoms. The mice were inoculated with different dosages of HSV-1 17syn+ on scarified corneas and recorded for survival rates, body weights, and disease scores on the indicated days after infection. n=6 mice. Data and error bars represent mean ± SEM.



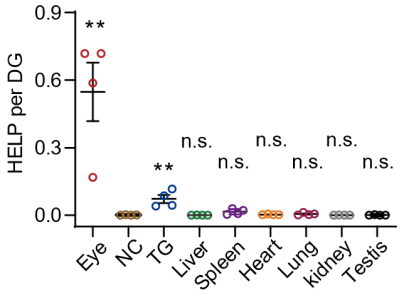
Supplementary Fig. 7 Time course of HSV-1 infection in the corneas. The mice were inoculated with 2×10^6 PFU HSV-1 17syn+ on scarified corneas. Sections were prepared on 12 hours post infection, 2, 5, and 8 days post infection, respectively. hpi, hour post infection; dpi, days post infection. The experiment was repeated twice with similar results.



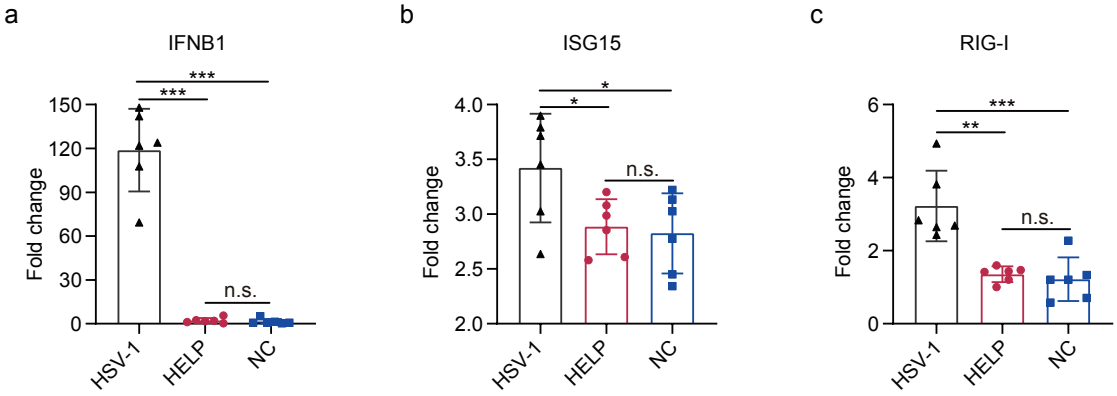
Supplementary Fig. 8 Intrastromal injection of mouse corneas. a, An eye of C57BL/6J mouse before injection; **b** and **c**, A small pocket was created in the mid-peripheral cornea using a 29G needle. **d**, Injection of PBS with a 33G needle. **e**, The eye 5 days after injection.



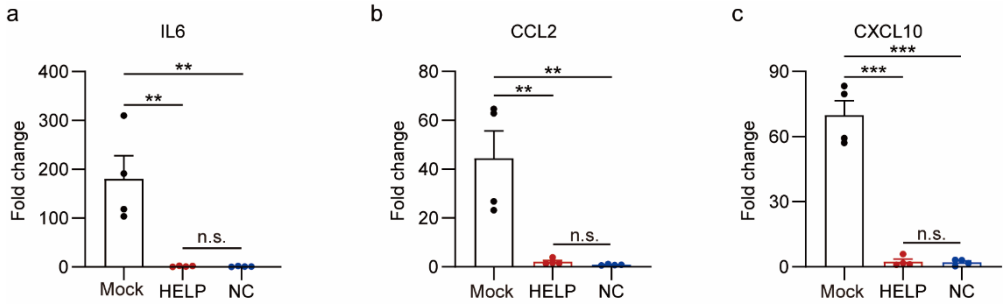
Supplementary Fig. 9 Western blot analysis of the life-span of Cas9 by HELP delivery. a. The life-span of Cas9 protein by HELP and lentivirus delivery *in vitro*. 293T cells were seeded 24 h before transduction at a density of 4×10^4/well. 100 ng p24 used for each vector. **b**. The life-span of Cas9 protein after intrastromal delivery of HELP. 100 ng p24 of HELP was used for each eye by intrastromal injection. The experiment was repeated twice with similar results.



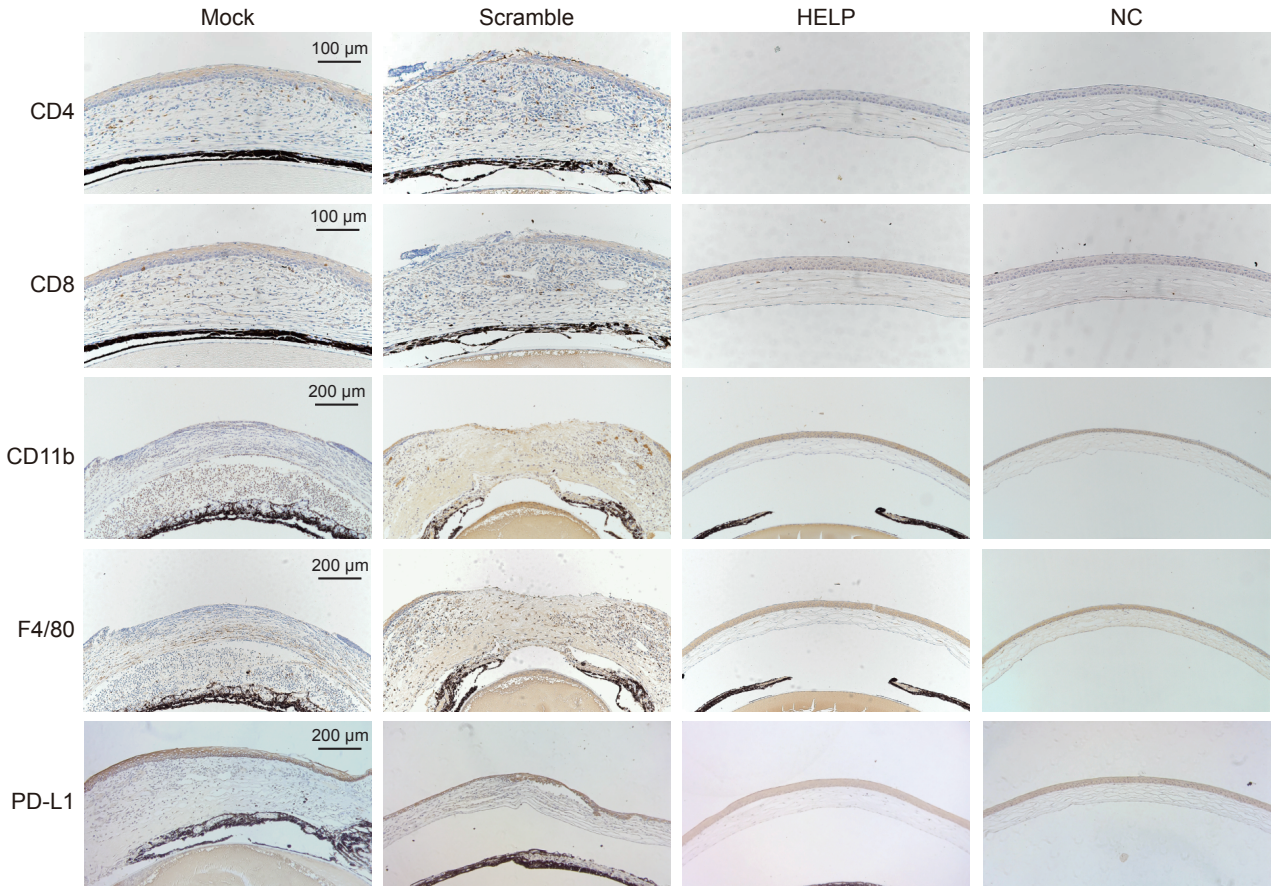
Supplementary Fig. 10 Tissue distribution of HELP in the whole body. qPCR quantification of HELP dissemination in different tissues as viral genome (VG) per diploid genome (DG) (n=4 mice). NC vs. Eye, *P*=0.0057; NC vs. TG, *P*=0.0077. Data and error bars represent mean ± SEM. Unpaired two-tailed Student's t-tests were performed. n.s.=non-significant.



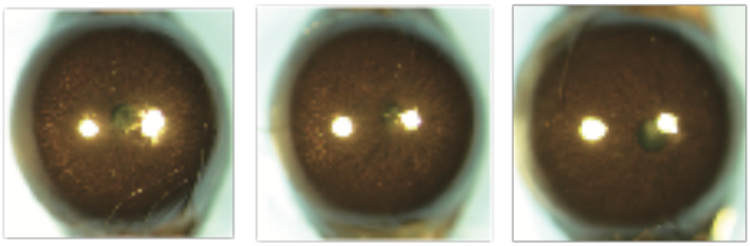
Supplementary Fig. 11 The innate immune response induced HELP and HSV-1 infection *in vivo*. Eyes were harvested for IFNB1 (**a**), ISG15 (**b**) and RIG-I (**c**) analysis by RT-qPCR 24 h after intrastromal injection of HSV-1 17syn+ (10^5 PFU) and 100 ng p24 HELP, respectively (n=5 mice). **a**, *P*<0.0001. **b**, HSV-1 vs. HELP, *P*=0.0399; HSV-1 vs. NC, *P*=0.0395. **c**, HSV-1 vs. HELP, *P*=0.0015; HSV-1 vs. NC, *P*=0.0009. Data and error bars represent mean ± SEM. Unpaired two-tailed Student's t-tests were performed. n.s.=non-significant.



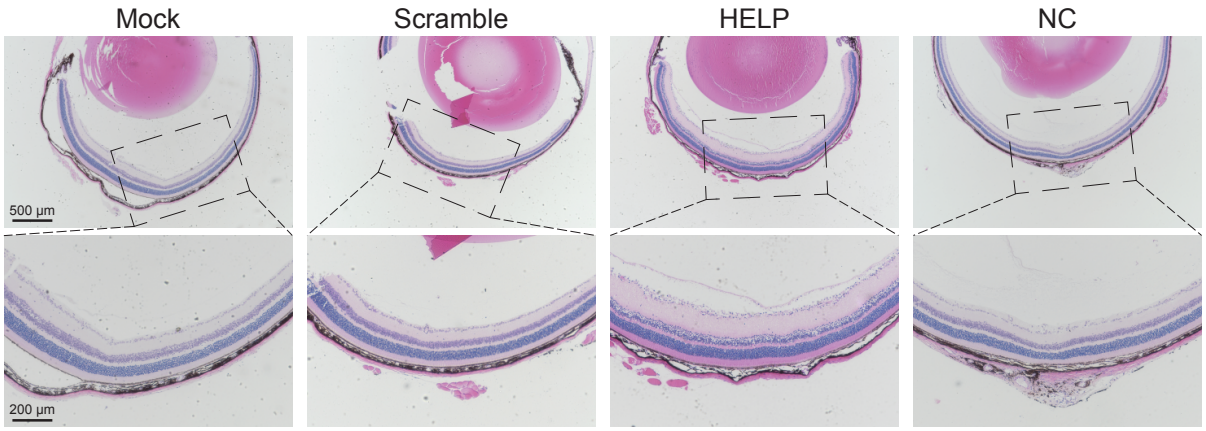
Supplementary Fig. 12 Inflammatory cytokines expressed in the corneas following HSV-1 17syn+ infection. Corneas were processed for RT-PCR analysis of inflammatory molecules (IL6, CCL2, and CXCL10) two weeks after HSV-1 17syn+ infection (2×10^6 PFU) of corneas (n=4 mice). **a**, Mock vs. HELP, *P*=0.0089; Mock vs. NC, *P*=0.0088. **b**, Mock vs. HELP, *P*=0.0093; Mock vs. NC, *P*=0.0081. **c**, *P*<0.0001. Data and error bars represent mean ± SEM. Unpaired two-tailed Student's t-tests were performed. n.s.=non-significant.



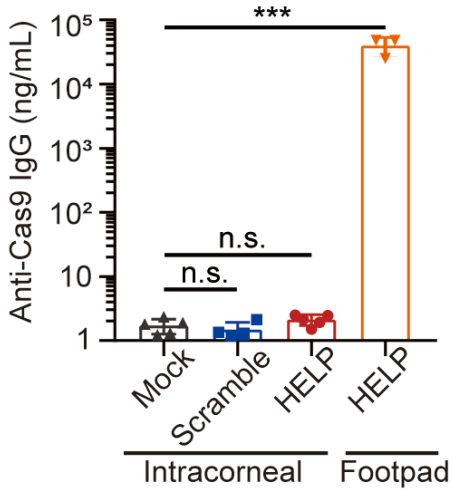
Supplementary Fig. 13 Immune cells infiltration and PD-L1 expression in the corneas *in vivo*. The corneas of mice were treated with HELP one day before infection with HSV-1 17syn+ (2×10^6 PFU). Immunohistochemistry analysis of $CD4^+$, $CD8^+$ cells, myeloid-derived cells ($CD11b^+$), and macrophages ($F4/80^+$) infiltration as well as PD-L1 expression in the corneas 14 days after infection. n=2 mice for each group. The experiment was repeated once. Scale bars, 100 μm or 200 μm.



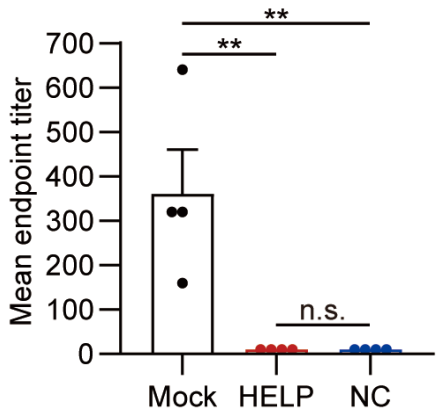
Supplementary Fig. 14 Long term follow-up of HELP treated mice in the preventive model. The mice were treated with HELP before inoculation with 2×10^6 PFU HSV-1 17syn+ on scarified corneas. Representative graphs show eyes from three different mice that have been followed up for three months.



Supplementary Fig. 15 Histological analysis of the retina of mouse eyes. The corneas of mice were treated with HELP one day before infection with HSV-1 17syn+ (2×10^6 PFU). The histology of retina was analysed 14 days after infection. n=4 mice for each independent experiment. Scale bars, 500 µm or 200 µm.



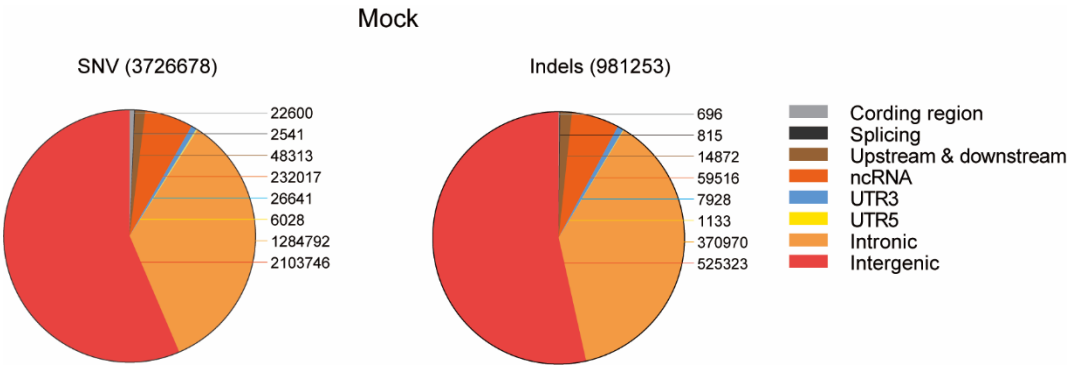
Supplementary Fig. 16 Cas9-specific IgG in the serum. Mouse sera were collected at 14 dpi and analysed for anti-Cas9 IgG induction by HELP and non-targeting (scramble) mLP (n=5 mice). 100 ng p24 HELP and scramble or 2 µL PBS were injected into mice cornea by intrastromal injection. Footpad injection of 100 ng p24 HELP used as a positive control (n=3 mice). Mock vs. HELP (Footpad injection), *P*=0.0004. Data and error bars represent mean ± SEM. Unpaired two-tailed Student's t-tests were performed. n.s.=non-significant.



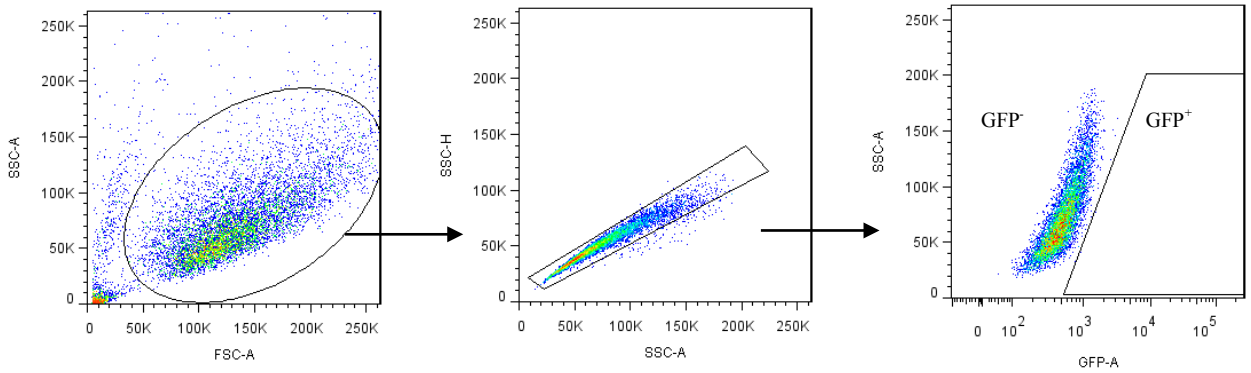
Supplementary Fig. 17 Neutralizing antibodies induced by HSV-1 infection. Mouse sera were collected at 28 dpi and analysed for HSV-1 neutralizing antibodies after HELP and Mock treatment. 100 ng p24 HELP or 2 µL PBS were injected into mice cornea by intrastromal injection. n=4 mice. *P*=0.0039. Data and error bars represent mean ± SEM. One-way ANOVA with Dunnett's multiple comparisons test. were performed. n.s.=non-significant.

Sample ID	HELP	MOCK
Total_Reads	1,059,941,298	938,567,089
Duplicates	160,681,499(15.16%)	136,392,833(14.53%)
Mapped_Reads	1,002,190,283(94.55%)	877,072,911(93.45%)
Properly_Mapped	979,842,046(92.44%)	856,870,912(91.3%)
PE_mapped	993,576,616(93.74%)	867,894,498(92.47%)
SE_mapped	1,368,621(0.13%)	2,862,324(0.3%)
With_Mate_Mapped_to_Diff_Chr	11,346,098(1.07%)	8,304,416(0.88%)
With_Mate_Mapped_to_Diff_Chr (mapQ>=5)	9,090,186(0.86%)	5,548,136(0.59%)
Mean_Sequencing_Depth	51.2459	44.5995
Coverage	92.38%	92.34%
Coverage_above_4X	92.3%	92.26%
Coverage_above_10X	91.85%	92.05%
Coverage_above_20X	90.73%	89.93%
Coverage_above_30X	86.24%	83.74%

Supplementary Fig. 18 The overall quality of WGS data. Quality of whole genome sequencing (WGS) data for HELP and Mock treated the human corneal punches which were derived from the same donor.



Supplementary Fig. 19 Identification of SNV and indel mutations in the Mock treated corneal punch at WGS level. Valid sequencing data were aligned to Human Genome version 19 (HG19).



Supplementary Fig. 20 Gating strategies used for flow cytometry analysis. Gating strategy used to analyse GFP^+ cells in HSV1-GFP infected primary mouse corneal stromal cells in Fig. 1k-1l.

Supplementary Table 1

Unique SNVs and indels in the HELP (UL8 gRNA) treated human cornea uncovered by whole genome sequencing.

HELP induced SNVs and indels (UL8 gRNA)

#CHRO	POS	REF	ALT	Func	Gene	MutType	DNA	crRNA
chr2	220873076	C	CA	intergenic	MIR4268;EPHA4	InDel	GGGGCAGCCAcACaGaGTgAGGG	GGGGCAGCCATACCGCGTAANGG
chr16	27851353	CTTT	C	intronic	GSG1L	InDel	GGGGCtGCCATcCCcCtTcAGGG	GGGGCAGCCATACCGCGTAANGG
chr17	22005942	C	T	intergenic	FLJ36000;MTRNR2L1	SNP	GGGGCAGCgAggCCGaGTgATGG	GGGGCAGCCATACCGCGTAANGG
chr1	115881954	C	T	intergenic	NGF;VANGL1	SNP	GtGGCAtCCATACaGgGTAgGGG	GGGGCAGCCATACCGCGTAANGG
chrX	18413417	G	C	intergenic	SCML2;CDKL5	SNP	GaGGCtGCCAaACCaCcTAAGGG	GGGGCAGCCATACCGCGTAANGG
chr11	11317799	G	A	intronic	GALNT18	SNP	GGaGaAGCCATgCCGCaTtAGGG	GGGGCAGCCATACCGCGTAANGG
chr10	134446406	C	T	intronic	INPP5A	SNP	GGGGCAttCAcACCtCGgAAGGG	GGGGCAGCCATACCGCGTAANGG
chr18	25540302	C	T	intronic	CDH2	SNP	GGGtCAGgCATACaGaGTAtGGG	GGGGCAGCCATACCGCGTAANGG
chr20	44842922	G	A	intronic	CDH22	SNP	GaGGCAGCCActCCaCGcAAGGG	GGGGCAGCCATACCGCGTAANGG
chr11	107263096	G	A	intronic	CWF19L2	SNP	GGGGtAtCCATACacaGTAATGG	GGGGCAGCCATACCGCGTAANGG
chr11	107263213	A	G	intronic	CWF19L2	SNP	GGGGtAtCCATACacaGTAATGG	GGGGCAGCCATACCGCGTAANGG
chr11	107263150	A	G	intronic	CWF19L2	SNP	GGGGtAtCCATACacaGTAATGG	GGGGCAGCCATACCGCGTAANGG
chr13	39592564	C	A	intronic	PROSER1	SNP	GtGaCAGgCATACaGtGTAAAGG	GGGGCAGCCATACCGCGTAANGG
chr2	44973791	A	T	intronic	CAMKMT	SNP	GGtGCAGCCATACCtgcaAAAGG	GGGGCAGCCATACCGCGTAANGG
chrX	152908152	A	G	intronic	DUSP9	SNP	GGGGCAGCCAggCCGgGTggGGG	GGGGCAGCCATACCGCGTAANGG
chr1	14925003	C	T	upstream	KAZN	SNP	GGGGCtGCCccACCGCtcAAGGG	GGGGCAGCCATACCGCGTAANGG

Supplementary Table 2

Unique SNVs and indels in the HELP (UL29 gRNA) treated human cornea uncovered by whole genome sequencing.

HELP induced SNVs and indels (UL29 gRNA)

#CHRO	POS	REF	ALT	Func	Gene	MutType	DNA	crRNA
chr9	132279711	T	TTTTC	intergenic	LINC00963;NTMT1	InDel	cgGAGtGTACtCGTtTCCCAGG	GCGAGCGTACACGTATCCCNGG
chrX	38942374	GA	G	intergenic	MID1IP1;LINC01281	InDel	GtGAGCcTgCACcTtTCCCTGG	GCGAGCGTACACGTATCCCNGG
chr2	27051725	CAAA	C	intergenic	CENPA;DPYSL5	InDel	cCcAGtGTtCACtTATCCCAGG	GCGAGCGTACACGTATCCCNGG
chr11	1554083	GC	G	intergenic	MOB2;DUSP8	InDel	GCcAGCGcACACGgcTCCCTGG	GCGAGCGTACACGTATCCCNGG
chr7	88796758	T	TATAA	intronic	ZNF804B	InDel	GCGAGaGTtCACtTATaCaGGG	GCGAGCGTACACGTATCCCNGG
chr9	120670834	C	A	intergenic	TLR4;BRINP1	SNP	ctGAGCcTACACaTtTCCCTGG	GCGAGCGTACACGTATCCCNGG
chr5	1687054	G	A	intergenic	LOC728613;MIR4277	SNP	GgGAGaGTACttGgATCCCAGG	GCGAGCGTACACGTATCCCNGG
chr7	27362607	G	A	intergenic	EVX1;HIBADH	SNP	GCGAatGTACAgtTgTCCCTGG	GCGAGCGTACACGTATCCCNGG
chr10	106298543	A	G	intergenic	LOC101927523;SORCS3	SNP	GCGAGgGTACAtGataCCCAGG	GCGAGCGTACACGTATCCCNGG
chr8	59617284	T	A	intergenic	NSMAF;TOX	SNP	GaGAGCacACACaTtTCCCTGG	GCGAGCGTACACGTATCCCNGG
chr1	235056669	A	G	intergenic	LINC01132;LOC101927851	SNP	GtGAGCacACACaTAgCCCGGG	GCGAGCGTACACGTATCCCNGG
chr17	77924776	C	T	intronic	TBC1D16	SNP	GCGAGCagtCACGaATCCaGGG	GCGAGCGTACACGTATCCCNGG
chr11	34532204	T	C	intronic	ELF5	SNP	GCctGtGTtCACaTATCCCTGG	GCGAGCGTACACGTATCCCNGG
chr7	157437264	C	T	intronic	PTPRN2	SNP	GCcAGCaTACgCcTcTCCCAGG	GCGAGCGTACACGTATCCCNGG
chr11	7646378	T	C	intronic	PPFIBP2	SNP	GtGAGCGTgCACacATgCCTGG	GCGAGCGTACACGTATCCCNGG
chr2	238789202	T	C	intronic	RAMP1	SNP	GtGgGtGTACACacATCCCAGG	GCGAGCGTACACGTATCCCNGG
chr19	4406160	G	A	intronic	CHAF1A	SNP	GgGAGCGTAgcaGcATCCCTGG	GCGAGCGTACACGTATCCCNGG
chr4	151170387	G	A	intronic	DCLK2	SNP	GaGAGgGcACAgGTcTCCCGGG	GCGAGCGTACACGTATCCCNGG
chr7	88796887	T	C	intronic	ZNF804B	SNP	GCGAGaGTtCACtTATaCaGGG	GCGAGCGTACACGTATCCCNGG
chr9	80911421	G	T	upstream	PSAT1	SNP	GCGgGCaTcCACGctTCCCAGG	GCGAGCGTACACGTATCCCNGG
chr14	104002611	T	C	UTR3	TRMT61A	SNP	GgGcGCaTACAaGTAaCCCAGG	GCGAGCGTACACGTATCCCNGG
chr9	127962972	G	A	UTR5	RABEPK	SNP	GCGAGgGTcCcCGgATaCCGGG	GCGAGCGTACACGTATCCCNGG

Supplementary Table 3
gRNA sequences and primers used to amplify the target sites for TIDE analysis.

Target sites	gRNA Sequence (5'-3')	Primer names	Sequence (5'-3')
UL8	GGGGCAGCCATACCGCGTAA	Y1-F	gagccgtagaaatcccgcag
		Y2-R	aaacctcaccaaacagacaa
UL29	GCGAGCGTACACGTATCCC	Y3-F	gggtgtagtccgaaaagccaa
		Y4-R	cacgcccccaggtaaagtgta

Supplementary Table 4
Primers used in RT-PCR.

Primer names	Sequence (5'-3')
Y5-HSV-gD-F	tacaacctgaccatcgcttg
Y6-HSV-gD-R	gcccccagagacttgttgta
SK13-GAPDH(Mouse)-F	gtgttcctacccccaatgtg
SK14-GAPDH(Mouse)-R	tagcccaagatgcccttcag
SK9-WPRE-F	gtcctttccatggctgctc
SK10-WPRE-R	ccgaagggacgtagcaga
SK11-Cas9-F	cagattcgcctggatgacca
SK12-Cas9-R	atccgctcgatgaagctctg
SK55-GAPDH(Human)-F	tccactggcgtcttcacc
SK56-GAPDH(Human)-R	ggcagagatgatgacccttt
SK51-ISG15(Mouse)-F	caggacggtcttacccttcc
SK52-ISG15(Mouse)-R	aggctcgctgcagttctgtac
Y7-RIG1(Mouse)-F	gccctgtaccatgcaggttac
Y8-RIG1(Mouse)-R	agtcccaactttcgatggctt
Y9-IFNB1(Mouse)-F	agctccaagaaaggacgaaca
Y10-IFNB1(Mouse)-R	gccctgtaggtgaggttgat
Y11-ISG15(Human)-F	ggcagcgaactcatcttt
Y12-ISG15(Human)-R	cagcatcttcaccgtcag
Y13-RIG1(Human)-F	tggaccctacctacatcctg
Y14-RIG1(Human)-R	tggaccctacctacatcctg
Y15-IFNB1(Human)-F	ttcagtgtcagaagctcctgtgg
Y16-IFNB1(Human)-R	ctgcttaatctcctcagggatgtca
Y17-IL6(Mouse)-F	catgttctctgggaaatcgtg
Y18-IL6(Mouse)-R	ttctgcaagtgcatcatcg
Y19-CCL2(Mouse)-F	aggtgtcccaaagaagctgta
Y20-CCL2(Mouse)-R	tctggacccattccttcttg
Y21-CXCL10(Mouse)-F	gtcattttctgcctcatcctgct
Y22-CXCL10(Mouse)-R	ggattcagacatctctgctcatca
Y23-GAPDH(Mouse)-F	aggtcggtgtgaacggatttg
Y24-GAPDH(Mouse)-R	ggggtcgttgatggcaaca

Supplementary Table 5
Primers used to amplify the on-target and the predicted off-target sites for NGS.

On- and off-targets	gRNA Sequence (5'-3')	Primer names	Sequence (5'-3')
UL8 On-target	GGGGCAGCCATACCGCGTAA	Y1-F	gagccgtagaaatcccgcag
		Y2-R	ggacaccgcagatatcgtgt
UL8 Off-target-Mus 1	GGCGATGCCAAACCGCGTAA	Y29-F	cccacagagccacgttcttc
		Y30-R	gtcagagtttaggagcacacc
UL8 Off-target-Mus 2	GGCAAAGCCATACCCCGTAA	Y31-F	agaagccgagtgaggaaagc
		Y32-R	gctgtaaacacctgagtgga
UL8 Off-target-Mus 3	GCAGCAGCCATACCGCCTCA	Y33-F	cagagtgggcagacagatagg
		Y34-R	gtctcgtccctcatttaacgc
UL8 Off-target-Mus 4	AGGGCAGCCATACCCCCTGA	Y35-F	ccaaccctactgcactgagc
		Y36-R	agagtccagagtgttccatg
UL8 Off-target-Mus 5	GGGGCAGGCATAGAGCATAA	Y37-F	tggagggaagaaatacgtgc
		Y38-R	gagagggagagaaggttgatt
UL8 Off-target-Mus 6	GGGGCTGCCATACAGAATAA	Y39-F	tccacctgtggcctctatac
		Y40-R	cttcttgtgtctcgcttgcc
UL29 On-target	GCGAGCGTACACGTATCCC	Y3-F	cgccgacagtaacgccagaa
		Y4-R	gtttgcggaccgattgccag
UL29 Off-target-Mus 1	GCGAGCGCACAAGGATCCC	Y53-F	ggaccagcagaagtgagtacg
		Y54-R	ggtggcttgatgcccaatga
UL29 Off-target-Mus 2	GCGAGCACTCACATATCCC	Y55-F	tccagatcctgctgcccttg
		Y56-R	cctgggtttcatggtacatgcc
UL29 Off-target-Mus 3	GAGAGTGTTCAAGTATCCC	Y57-F	gaggtagcacaagattgcaaa
		Y58-R	ccacgtttgctccagcctta
UL29 Off-target-Mus 4	GGCGAGCTTAGACGTTTTCC	Y59-F	ttgctttgtttatgtttgct
		Y60-R	ttacaatggacaagcccagg
UL29 Off-target-Mus 5	GTGAGACTACACGTCTCCCC	Y61-F	acaacaaggctcagggaatg
		Y62-R	catgctgttggctaaggaga
UL29 Off-target-Mus 6	GCGCGCGCCCCCGTATCCC	Y63-F	gaagcccagactccgcaggt
		Y64-R	tgggaagtcttcgacgtgtg