

SUPPLEMENTARY INFORMATION

Gnostic and agnostic immunotherapy by tropism-retargeted herpes simplex virus without direct tumor treatment

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SUPPLEMENTARY MATHERRIALS AND METHODS

Oligonucleotides sequences for TK gene insertion

name	Sequence
TK_F	AAAAGGATCCACCAATGGCTTCGTACCCCTGCCATCAAC
TK_R	TTTTGGATCCTCAGTTAGCCTCCCTCCC

Engineering of R-375. R-375 was derived from R-337 by GalK recombineering ^{1, 2}. To delete a portion of the first copy of the LAT sequence, the first recombination was performed with the galK cassette amplified with the oligonucleotides LAT1_galK-F and LAT1_galK-R; then the DNA for the second recombination was obtained by annealing and extension of oligos LAT1_collasso-F and LAT1_collasso-R. To delete a portion of the second copy of the LAT sequence, the first recombination was performed with the galK cassette amplified with the oligonucleotides LAT2_galK-F and LAT2_galK-R; then the DNA for the second recombination was obtained by annealing and extension of oligos LAT2_collasso-F and LAT2_collasso-R. To delete α -47 ORF, the first recombination was performed with the galK cassette amplified with the oligonucleotides D47_galK_F and D47_galK_R; then the DNA for the second recombination was obtained by annealing and extension of oligos D47_collasso_F and D47_collasso_R. In the US1/US2 locus, to substitute the mIL12 p70 previously introduced in R-337 ³ with the HA-CT26-16pep cassette, the first recombination was performed with the galK cassette amplified with the oligonucleotides US1/US2_galK_f and US1/US2_galK_r, then the second recombination was performed with the DNA obtained amplifying the pCMV-HA-CT26-16pep-bGHpolyA cassette on the plasmid pCDNA3.1(-)-HA-CT26-16pep with the oligonucleotides US1/US2_pCMV_f and US1/US2_polyA_r. To introduce mIL12 p70 into the UL26/UL27 intergenic locus, the first recombination was performed with the galK cassette amplified with the oligonucleotides UL26galkF and UL27galkR, then the second recombination was performed with the DNA obtained amplifying the pCMV-mIL12 p70-bGHpolyA cassette on the plasmid pCDNA3.1(-)-mIL12linker ⁴ with the

oligonucleotides UL26_CMV_f and UL27_pA_rev. R-371 was generated from R-375 by deleting the HA-CT26-16pep cassette from the US1/US2 locus. The first recombination step was carried out using the previously described D47_galK_F and D47_galK_R amplicon, while the second recombination was performed using the self-annealed US1/US2_collasso_F and US1/US2_collasso_R oligonucleotides. Western blot and immunofluorescence were described in ⁵.

Oligonucleotide sequences for R-375 and R-371 engineering

name	Sequence
LAT1_galK-F	CGGCCCCGGGCTGCCTGACCACCGATCCCCGAAAGCATCCTGCC ACTGGCACCTGTTGACAATTAATCATCGGCA
LAT1_galK-R	GGCGTGGCTGCCGGGAGGGGCCGCGGATGGGCGGGGCCTACT CGGTCTCCTCAGCACTGTCCTGCTCCTT
LAT1_collasso-F	CGGCCCCGGGCTGCCTGACCACCGATCCCCGAAAGCATCCTGCC ACTGGCAGGAGACCGAGTAGGCCCCGC
LAT1_collasso-R	GGCGTGGCTGCCGGGAGGGGCCGCGGATGGGCGGGGCCTACT CGGTCTCCTGCCAGTGGCAGGATGCTTT
LAT2_galK-F	AGAACCACAGTGGGTTGGGTGTGGGTGTTAAGTTTCCGCGAGC GCCTGCCCCCTGTTGACAATTAATCATCGGCA
LAT2_galK-R	TGCTCTTTCCCCGTGACACCCGACGCTGGGGGGCGTGGCTGCC GGGAGGGTCAGCACTGTCCTGCTCCTT
LAT2_collasso-F	AGAACCACAGTGGGTTGGGTGTGGGTGTTAAGTTTCCGCGAGC GCCTGCCCCCTCCCGGCAGCCACGCCC
LAT2_collasso-R	TGCTCTTTCCCCGTGACACCCGACGCTGGGGGGCGTGGCTGCC GGGAGGGGGCAGGCGCTCGCGGAAACT
D47_galK_F	GAACCACCCTCCGCCCAGAACTTGGGCGATGGTCGTACCCGG GACTCAACCTGTTGACAATTAATCATCGGCA
D47_galK_R	CCTGCTCGTCGGGGCGACCGGCGGCGACCGTTGCGTGGACCGC TTCCTGCTCAGCACTGTCCTGCTCCTT
D47_collasso_F	GAACCACCCTCCGCCCAGAACTTGGGCGATGGTCGTACCCGG GACTCAAGCAGGAAGCGGTCCACGCAACGG
D47_collasso_R	CCTGCTCGTCGGGGCGACCGGCGGCGACCGTTGCGTGGACCGC TTCCTGCTTGAATCCCGGTACGACCATCGC
US1/US2_galK_f	ATAAAAGACCAAAATCAAAGCGTTTGTCCCAGCGTCTTAATGG CGGGAAGCCTGTTGACAATTAATCATCGGCA
US1/US2_galK_r	AATAAACCCCCAAACACCCCCCATGTACGCGTGGTCTGTTTCTC TCCGCCTCAGCACTGTCCTGCTCCTT
US1/US2_pCMV_f	ATGTCCCCAAATAAAAGACCAAAATCAAAGCGTTTGTCCCAGC GTCTTAATGGCGGGAAGCGTTTTGCGCTGCTTCGCGATGTACG GGC
US1/US2_polyA_r	CCCCGATGTCAATAAACCCCCAAACACCCCCCATGTACGCGTG GTCTGTTTCTCTCCGCCGCCATAGAGCCCACCGCATCCCCAGCA TGCCTG
UL26galkF	CGGGTAGGCGGGTAGCTTTACAATGCAAAAGCCTTCGACGTGG AGGAAGGCCTGTTGACAATTAATCATCGGCA
UL27galkR	TTCCCGTGACAAAACGGACCCCTTGGTCAGTGCCGATTCCCCC CCCACGTCAGCACTGTCCTGCTCCTT

UL26_CMV_f	TTGGGAGGCGCGGGTAGGCGGGTAGCTTTACAATGCAAAAGCC TTCGACGTGGAGGAAGGCGATGTACGGGCCAGATATACGC
UL27_pA_rev	CCTCTTTCCTTTCCCGTGACAAAACGGACCCCCTTGGTCAGTGC CGATTCCCCCCCCACGGCCATAGAGCCCACCGCATC
USI/US2_collasso_F	CCTTGTATGTCCCCAAATAAAAAGACCAAAATCAAAGCGTTTGT CCCAGCGTCTTAATGGCGGGAAGGGCGGAGAGAAACAGACCA CGCG
USI/US2_collasso_R	CCGATGTCAATAAACCCCCAAACACCCCCCATGTACGCGTGGT CTGTTTCTCTCCGCCCTTCCCGCCATTAAGACGCTGGGACAAAC GC

Generation of pCDNA3.1(-)-HA-CT26-16pep plasmid. The synthetic gene encoding HA-CT26-16pep (see nucleotide sequence below) was cloned into pCDNA using NcoI and BamHI to generate pCDNA3.1(-)-HA-CT26-16pep, which served as a template for the PCR employed in the recombineering protocol.

Sequence:ccatgggacactctggacagaaccacctgaaagaaatggccatcagcgtgctggaagccagagcctgtgctgctgctggaca
gtctggctctggaatcctgcctcaggctcctagcggacctagctacgccacatatctgcagccagctcaggcccagatgctgacacctcttgaa
gcggccacagcttcacacgctgctatgggcatggcgtgacttgggtgcgcgccatcatgacaaaggccagctacagctctggcagcggc
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ccggcaagatgaagctcgagatcagcgaagctctggcccctggcacaagtggctctggcatcacaacatgtctggctgtcgggcgcctggac
gtgaagttaacagaggctgctctgagagccgctcctgacatcctgatttccggatctggcggagaggtgccacctcagaactgcaggctctgc
aaagagccctgcagagcgagttctgcaacgccgtcagagaggtgtactccggaagcggctacccttacgactgccagattacgcctgagga
tcc

Re-IP regimen, combination with anti-CTLA-4 Abs, depletion of CD4+ and CD8+ cells, immune reactivity test. For the Re-IP regimen, 2-9 x 10⁶ CT26-HER2-TK cells were infected *ex vivo* with the R-375 virus at a multiplicity of infection of 3. After 4 hours, the infected cells were trypsinized, washed twice with medium, resuspended in 100 µl of serum-free medium, and injected intraperitoneally (i.p.) into the mice. Vehicle mice were injected with 100 µl of Phosphate Buffer Saline (PBS). Where indicated, the following treatments were administered intraperitoneally: 70 µg anti-CTLA-4 (αCTLA4) in 100 µL of PBS; 200 µg anti-CD4 (αCD4) or anti-CD8 (αCD8) in 100 µL PBS; 750 µg acyclovir in 500 µL of 0.9% NaCl solution; Renca lysate, obtained by lysing 3x10⁶ cells by sonication in 100 µl of PBS. The procedures for mice immunization against HSV-1, as well as the determination of anti-HSV-1 antibodies and neutralizing antibodies titers were previously described ⁵. CD4 and CD8 depletion was confirmed by flow cytometry on isolated splenocytes ⁶. The reactivity of splenocytes and sera against wt-CT26, CT26-HER2, and Renca cells was determined as previously described ⁷.

Ex vivo analyses. To determine active HSV-1 infection, total RNA was extracted from tumor samples, retrotranscribed to cDNA, and analyzed by qRT-PCR for gC mRNA⁵. Expression levels of *Ly75*, *Il12*, *Tbet21*, *Cxcl9*, and *Ccl5* were also measured on these cDNAs using specific TaqMan probes, normalized to *Rpl13a*, and expressed as log2 fold changes relative to the mean values of the Vehicle group. A peritoneal wash was performed by injecting 2 ml PBS into the peritoneal cavity of mice post-mortem and retrieving the injected solution. From this, 1 ml of the peritoneal wash was centrifuged at 3000 g for 5 minutes to collect cells, which were then washed twice with 1 ml PBS, stained for human-HER2, and analyzed by flow cytometry⁷. Another 1 ml of the peritoneal wash was sonicated for 10 s on ice and plated on SK-OV-3 monolayers (6×10^6 cells per 3.5 cm^2 area) to determine HSV PFUs. After 120 minutes incubation, the inoculum was removed, the monolayers were covered with an agarose overlay, and plaque number was scored after 5 days. Purified virions were titrated in parallel as a positive control. The peritoneal cavity was sampled by collecting 10 small peritoneal specimens (a few milligrams each). These specimens were mixed and processed to obtain a single-cell suspension, which was then stained for human-HER2 and analyzed by flow cytometry following the same protocol used for tumor processing and staining in⁷.

SUPPLEMENTARY FIGURES

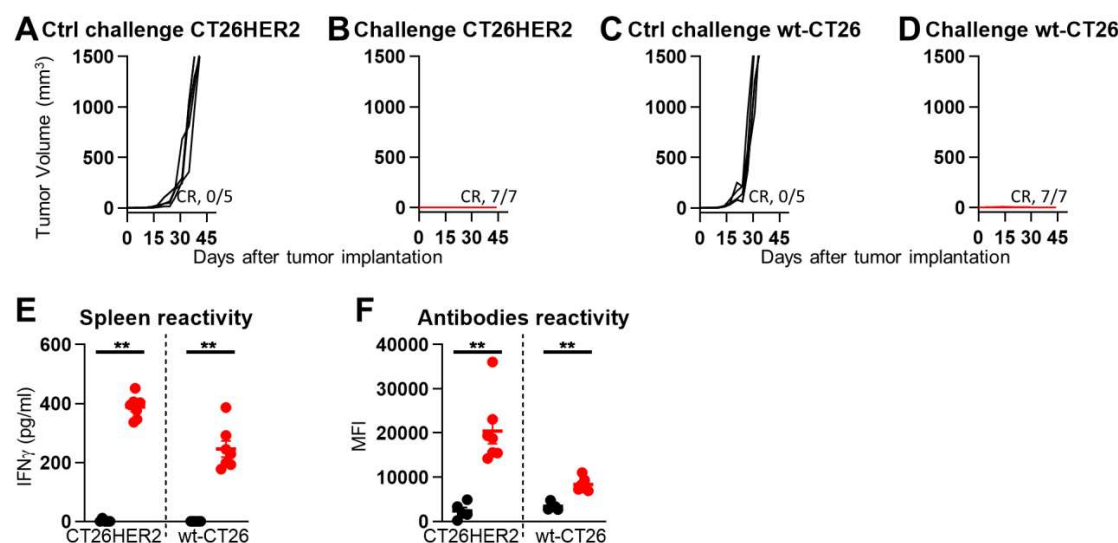


Fig. S1. Long-term protection by Re-IP. Mice from the experiments shown in Figures 2 and 3 that underwent CR to both wt-CT26 and CT26-HER2 tumors were rechallenged on day 47 post-primary tumor engraftment by implanting CT26-HER2 and wt-CT26 challenge tumors (1×10^6 cells). The kinetics of CT26-HER2 or wt-CT26 tumor growth in the control group (A, C) and Re-IP CR group (B, D) are shown. Control group: $n=5$; challenge group from CR Re-IP: $n=7$ (2 mice from the experiment in Fig. 2 and 5 mice from the experiment in Fig. 3). (E, F) Immune response (see legend to Fig. 1) quantified as spleenocyte reactivity (E) and serum reactivity (F). (E, F) Statistical significance was calculated using the Mann-Whitney test (Shapiro-Wilk test failed); comparisons were separately performed on CT26-HER2 and wt-CT26 cells.

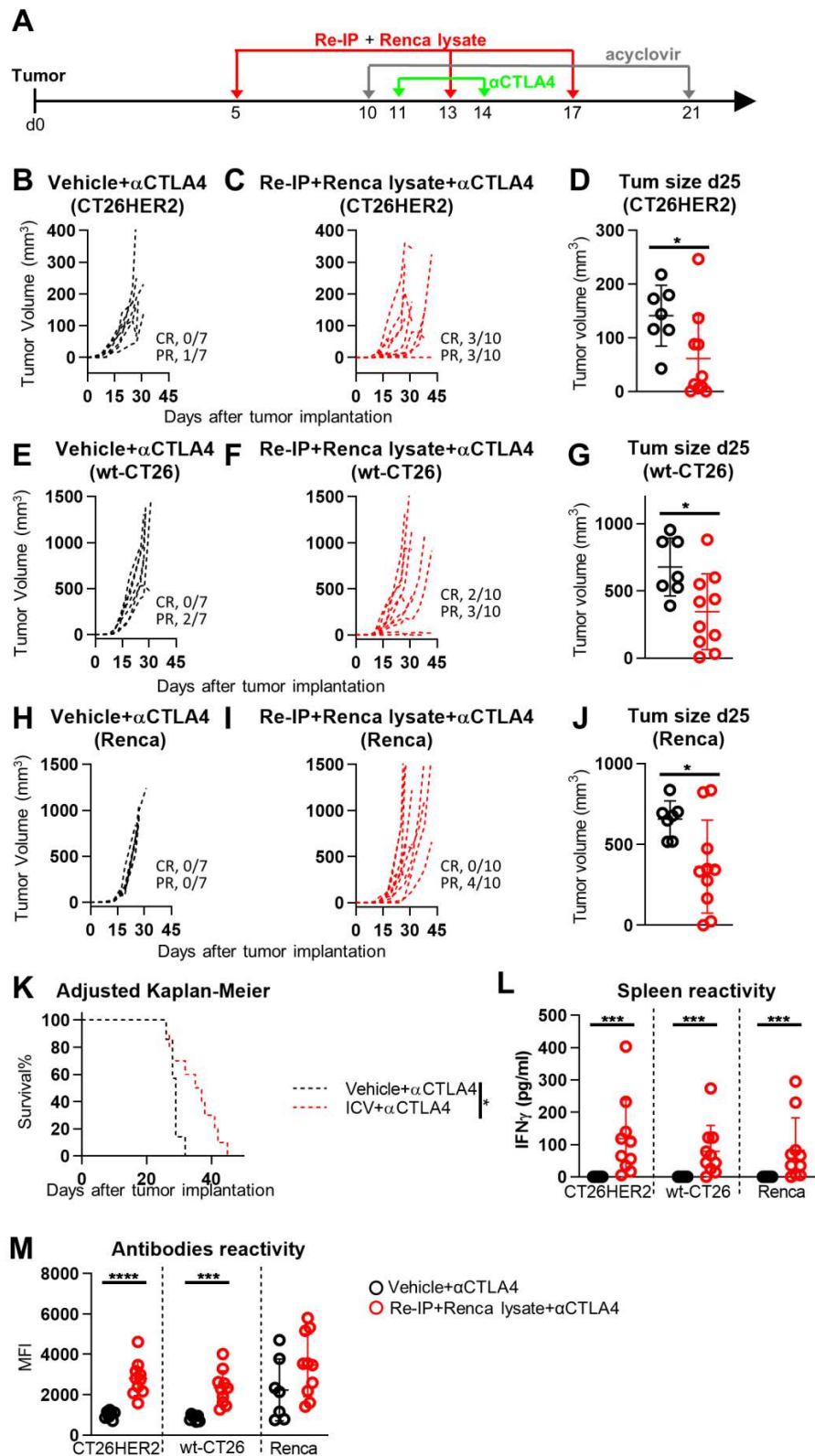


Fig. S2. Effect of combination of CT26-based Re-IP and Renca tumor cell lysate. (A) Regimen of treatment. On day 0, mice were implanted with CT26-HER2 and wt-CT26 tumors (3×10^5 cells) in the left and right flanks, and with Renca tumors (1×10^6 cells) in the back, respectively. On days

5, 13, and 17 post-tumor engraftment, mice received three i.p. doses of Re-IP (3×10^6 cells/injection) or vehicle, in combination with Renca cell lysate, acyclovir, and α -CTLA4. Vehicle group: n=7; Re-IP: n=10. (B, C) Kinetics of CT26-HER2 tumor growth, with CR and PR. (D) CT26-HER2 tumor volumes on day 25. (E, F) Kinetics of wt-CT26 tumor growth, with CR and PR. (G) wt-CT26 tumor volumes on day 25. (H, I) Kinetics of Renca tumor growth, with CR and PR. (J) Renca tumor volumes on day 25. (K) Kaplan-Meier survival curve adjusted for Renca tumors (adjusted Kaplan-Meier): only Renca tumors were considered for the graph and for mice that reached the endpoint due to wt-CT26 or CT26-HER2 tumors, the Renca tumor growth curve was fitted to an exponential equation to estimate the Renca tumor endpoint, defined as the day when tumor volume exceeded 1500 mm³. (L, M) Immune reactivity towards the three types of tumors (see legend to Fig. 1), quantified as splenocyte response (L) and serum antibodies (M) to CT26-HER2, wt-CT26, and Renca cells. (D, G, J-M) Statistical significance was calculated using the Mann-Whitney test (Shapiro-Wilk test failed) (D, L), two-tailed t-test (Shapiro-Wilk test passed, F test passed) (G), Welch's test (Shapiro-Wilk test passed, F test failed) (J, M), Log-rank Mantel-Cox test (K). (L, M) Comparisons were performed on CT26-HER2, wt-CT26, and Renca cells separately.

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