Pre-induced reovirus-specific T-cell immunity enhances the anticancer efficacy of reovirus therapy

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Figure S1 Local and systemic presence of reovirus-specific T cells in the TC1 model. **(A)** Design of experiment described in B-C. Mice (n=5–8/group) with established TC1 tumors were intratumorally (i.t.) injected with reovirus (10^7 plaque-forming units (pfu)) on 3 consecutive days. Mice were sacrificed 7 days after the first reovirus injection for ex vivo analysis of tumors and spleens. **(B)** Frequency of $CD3^+$ and $CD8^+$ T cells within the total $CD45^+$ immune cell population in TC1 tumors after reovirus administration. **(C)** Frequency of interferon γ $(IFN\gamma)^+$ cells within the intratumoral and splenic $CD8^+$ T-cell population as measured with intracellular cytokine staining. Single-cell suspensions (n=5/group) were cocultured with indicated targets for 6 hours. Medium was used as negative control and PMA/ionomycin (IO) was used as positive control. Data are presented as mean±SEM. Statistical tests used: (B): unpaired t-test between PBS and Reo groups. (C): ordinary one-way analysis of variance (ANOVA) with Dunnett's post hoc test. Statistical difference was compared to medium control group. Significance levels: ***p<0.001 and ****p<0.0001.

Figure S2 MHC class I expression and reovirus replication in TC1, Fre.Kb and Fre.Db cells. (A) Kb and Db expression on TC1, Fre.Kb and Fre.Db cells as measured with flow cytometry. **(B)** Reovirus genomic segment 4 (S4) copy number in TC1, Fre.Kb and Fre.Db cells after reovirus infection. Cells (150000/well) were infected with multiplicities of infection (MOI)=10. Samples (n=2-3) were harvested 24 hours after infection and reovirus S4 copy numbers were determined by quantitative reverse transcription PCR (RT-qPCR). Individual data points represent 2-3 biological duplicates with each 2 technical replicates.

Figure S3 T-cell recognition of positive peptide pools. (A) Frequency of interferon γ (IFNγ)$^+$ cells within the reovirus-specific T-cell bulk as measured with intracellular cytokine staining. T cells were cocultured with peptide pools (1 μg/mL for each peptide) for 6 hours. Medium was used as negative control and phorbol 12-myristate 13-acetate (PMA)/ionomycin (IO) was used as positive control.

Figure S4 Frequency and distribution of reovirus-specific T cells in TC1-bearing mice. Quantification of Tm$^+$ cells out of CD8$^+$ T cells and total CD45$^+$ immune cell population in indicated organs on day 7 after the first intratumoral reovirus injection in mice bearing established TC1 tumors. Data are presented as mean±SEM.

Figure S5 Reovirus-specific T cells in tumors after intratumoral Reo or Jin-3 administration. Representative flow cytometry plots of Tm$^+$ CD8$^+$ T cells in tumors injected with Reo or Jin-3 according to the schedule described in Figure 2A. Tumors were harvested on day 7 after the first intratumoral reovirus injection.

Figure S6 cDC1 absence in *Batf3*$^{-/-}$ mice. **(A)** Representative flow cytometry plots of cDC1s (characterized by XCR1 and CD103 expression in tumors, and XCR1 and CD8 expression in other organs) in tumors, spleens, tumor-draining lymph nodes (TDLN), and blood of KPC3-bearing, PBS-treated C57BL/6J mice or *Batf3*$^{-/-}$ mice. **(B)** Quantification of cDC1s (n=5-7/group). All data are presented as mean±SEM.

Figure S7 Phenotype of reovirus-specific T cells on day 12. Expression of activation markers on Tm$^-$ or Tm$^+$ CD8$^+$ T cells in blood, spleen, tumor-draining lymph node (TDLN), and tumor, 12 days after the first intratumoral reovirus injection. All data are presented as mean±SEM.

Statistical tests used: (A): ordinary one-way analysis of variance (ANOVA) with Tukey's post hoc test. Significance levels: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Figure S8 Processing of SLPs by D1 cells and activation of reovirus-specific T cells. Frequency of IFNγ$^+$ cells within reovirus-specific T cell bulk after coculture with peptide #9 or the SLP (10 µM to 10 pM). Peptides were added directly or in the context of D1 cells as antigen-presenting cells and incubated with the reovirus-specific T cell bulk for 6 hours. Before coculture with T cells, D1 cells were pre-incubated for 1 hour with peptide #9 or SLP after which lipopolysaccharide (LPS; 10 µg/mL) was added to each well for an additional 23 hours.

Figure S9 Depletion of CD8 T cells diminishes SLP+Reo effect. **(A)** Design of experiment described in (B-D). Mice were vaccinated on days 0 and/or 14 by injecting 100 µg SLP together with 20 µg CpG in the tail-base region. On day 22, KPC3 tumor challenge was performed. 8 days after KPC3 tumor inoculation, CD8$^+$ T-cell depletion was initiated (Clone 2.42, 50 µg intraperitoneal). Mice with established KPC3 tumors were intratumorally (i.t.) injected with reovirus (10^7 plaque-forming units (pfu)) on days 13, 14, and 15 after tumor challenge. **(B)** Frequency of Reo µ1$_{133\text{-}140}$ Tm$^+$ cells within CD8$^+$ T cells after vaccination. **(C)** Frequency of CD8$^+$ T cells in blood after CD8$^+$ T-cell depletion. **(D)** Average growth curves of mice (n=10/group) receiving indicated treatments. All data are presented as mean±SEM. Statistical tests used: (C) unpaired t-test. (D): ordinary two-way ANOVA with Tukey's post hoc test. Significance levels: *p<0.05, **p<0.01, ****p<0.0001. Control group shared with Figure S12.

Figure S10 Irrelevant SLP vaccination impairs antitumor efficacy of SLP+Reo therapy. **(A)** Design of experiment described in (B-C). Mice were vaccinated with an SLP containing the reovirus epitope (Reo SLP) or an irrelevant SLP containing an HPV E7 epitope (E7 SLP)

on days 0 and 14 by injecting 100 µg SLP together with 20 µg CpG in the tail-base region. On day 22, KPC3 tumor challenge was performed. Mice with established KPC3 tumors were intratumorally (i.t.) injected with reovirus (10^7 plaque-forming units (pfu)) on days 12, 13, and 14 after tumor challenge. **(B)** Frequency of Reo $\mu1_{133-140}$ or HPV16 $E7_{49-57}$ Tm^+ cells within $CD8^+$ T cells after vaccination. **(C)** Average growth curves of mice (n=10/group) receiving indicated treatments. All data are presented as mean±SEM. Statistical tests used: (C): ordinary two-way ANOVA with Tukey's post hoc test. Significance level: **p<0.01. Control groups shared with Figure S13 and Figure 8.

Figure S11 Presence of reovirus-specific but not tumor-specific T cells in end-stage tumors after SLP+Reo therapy. **(A)** Separation of Tm^+ cells from Tm^- cells within total $CD8^+$ T cell population of end-stage KPC3 tumors after Reo or SLP+Reo therapy. **(B)** Frequency of interferon γ $(IFN\gamma)^+$ cells within the intratumoral $CD8^+$ T-cell population of end-stage KPC3 tumors that received Reo or SLP+Reo therapy. Single-cell suspensions (n=5/group) were cocultured with indicated targets. PMA/ionomycin (IO) was used as a positive control, and the irrelevant cell line TC1 was used as target cell line for reovirus infection. Data are presented as mean±SEM. Statistical tests used: (B): ordinary one-way analysis of variance (ANOVA) with Dunnett's post hoc test. Statistical difference was compared to medium control group. Significance level: ****p<0.0001.

Figure S12 The antitumor effect of SLP+Reo therapy cannot be improved by the addition of αPD-L1 therapy. **(A)** Design of experiment described in (B-C). Mice were vaccinated with an SLP containing the reovirus epitope on days 0 and 14 by injecting 100 µg SLP together with 20 µg CpG in the tail-base region. On day 22, KPC3 tumor challenge was performed. Mice with established KPC3 tumors were intratumorally (i.t.) injected with reovirus (10^7 plaque-

forming units (pfu)) on days 13, 14, and 15 after tumor challenge. αPD-L1 was administered intraperitoneally (i.p.) on days 14, 16, and 19. **(B)** Frequency of Reo $\mu1_{133\text{-}140}$ Tm^+ cells within $CD8^+$ T cells after vaccination. **(C)** Average growth curves of mice (n=10/group) receiving indicated treatments. All data are presented as mean±SEM. Statistical tests used: (C): ordinary two-way ANOVA with Tukey's post hoc test. Significance level: **p<0.01. Control group shared with Figure S9.

Figure S13 Continuation of intratumoral reovirus administration does not improve the antitumor effect of SLP+Reo therapy. (**A**) Design of experiment described in (B-C). Mice were vaccinated with an SLP containing the reovirus epitope on days 0 and 14 by injecting 100 µg SLP together with 20 µg CpG in the tail-base region. On day 22, KPC3 tumor challenge was performed. Mice with established KPC3 tumors were intratumorally (i.t.) injected with reovirus (10^7 plaque-forming units (pfu)) on days 12, 13, and 14 after tumor challenge. One group continued to receive intratumoral reovirus injections every 2 days after day 14. **(B)** Frequency of Reo $\mu1_{133\text{-}140}$ Tm^+ cells within $CD8^+$ T cells after vaccination. **(C)** Average growth curves of mice (n=10/group) receiving indicated treatments. All data are presented as mean±SEM. Statistical tests used: (C): ordinary two-way ANOVA with Tukey's post hoc test. Significance levels: *p<0.05, **p<0.01. Control groups shared with Figure S10 and Figure 8.

Figure S14 Boosting the reovirus-specific T cell response does not affect body weight. Increase in body weight (%) starting from the moment of the first intratumoral reovirus injection.

Supplementary Tables

Table S1. List of antibodies used for flow cytometric analysis.

	Marker	Clone	Fluorochrome	Supplier
Lymphoid panel	**CD45.2**	104	FITC	eBioscience
	CD3	145-2C11	PE-CF594	BD Biosciences
	CD8α	53-6.7	Alexa Fluor 700	eBioscience
	Tetramer		APC	In house
	CD44	IM-7	BV785	BioLegend
	CD62L	MEL-14	BV421	BioLegend
	NK1.1	Pk136	BV650	BD Biosciences
	PD-1	29F.1A12	APC-Cy7	BioLegend
	TIM-3	RMT3-23	PE	BioLegend
	NKG2A	16A11	PE	eBioscience
	KLRG-1	2F1	PE-Cy7	eBioscience
	CD103	2E7	BV711	BioLegend
	CD69	H1.2F3	BV605	BioLegend
Myeloid panel	**CD45.2**	104	FITC	BioLegend
	CD11b	M1/70	PE-Cy7	BioLegend
	CD11c	N418	APC-Cy7	BioLegend
	CD8α	53-6.7	Alexa Fluor 700	eBioscience
	CD103	2E7	BV711	BioLegend
	XCR1	ZET	PE	BioLegend
	CD4	RM4-5	APC	BioLegend
Intracellular cytokine staining panel	**CD45.2**	104	FITC	eBioscience
	CD3	145-2C11	PE-CF594	BD Biosciences
	CD8α	53-6.7	Alexa Fluor 700	eBioscience
	IFNγ	XMG1.2	APC	BioLegend

Table S2. GenBank accession numbers of Reovirus Type 3 Dearing isolate R124 segments.

Reovirus segment	**GenBank accession number**	**Link**	**Reference**
Segment S1	GU991665	https://www.ncbi.nlm.nih.gov/nuccore/325112732	[1]
Segment S2	GU991666	https://www.ncbi.nlm.nih.gov/nuccore/325112734	
Segment S3	GU991667	https://www.ncbi.nlm.nih.gov/nuccore/325112736	
Segment S4	GU991668	https://www.ncbi.nlm.nih.gov/nuccore/325112738	
Segment M1	GU991662	https://www.ncbi.nlm.nih.gov/nuccore/325112726	
Segment M2	GU991663	https://www.ncbi.nlm.nih.gov/nuccore/406601112	
Segment M3	GU991664	https://www.ncbi.nlm.nih.gov/nuccore/325112730	
Segment L1	GU991659	https://www.ncbi.nlm.nih.gov/nuccore/325112720	
Segment L2	GU991660	https://www.ncbi.nlm.nih.gov/nuccore/325112722	
Segment L3	GU991661	https://www.ncbi.nlm.nih.gov/nuccore/325112724	

1. van den Wollenberg, D.J., et al., *Isolation of reovirus T3D mutants capable of infecting human tumor cells independent of junction adhesion molecule-A.* PLoS One, 2012. **7**(10): p. e48064.

Table S3. Predicted H2-K^b reovirus epitopes tested in intracellular cytokine staining.

N	Peptide	Allele	nM	Rank	Segment	
1	ISDVYAPL	H-2-Kb	4.2	0.010	M1	
2	SAVLFSPL	H-2-Kb	3.9	0.010	L3	
3	MVYDYSEL	H-2-Kb	5.9	0.015	S4	Pool #1
4	SSYAWFIL	H-2-Kb	6.0	0.015	L1	
5	ISPAHAYL	H-2-Kb	7.4	0.020	M3	
6	LMYKYMPI	H-2-Kb	6.6	0.020	L2	
7	INFVSAML	H-2-Kb	8.3	0.025	M3	
8	LSLNFVTGL	H-2-Kb	10.5	0.030	S1	Pool #2
9	VSPKYSDL	H-2-Kb	10.9	0.030	M2	
10	VSYSGSGL	H-2-Kb	13.3	0.040	S1	
11	ISITSAAL	H-2-Kb	14.0	0.040	M3	
12	AVQLFRPL	H-2-Kb	14.3	0.040	L2	
13	VAVQLFRPL	H-2-Kb	14.2	0.040	L2	Pool #3
14	QGYYMAQL	H-2-Kb	14.1	0.040	L1	
15	VNPYYRLM	H-2-Kb	17.4	0.050	L2	
16	SNQAFYDLL	H-2-Kb	15.9	0.050	L2	
17	VGYLQYPM	H-2-Kb	17.2	0.050	L1	
18	LNANYFGHL	H-2-Kb	18.6	0.060	M1	Pool #4
19	KSRLRYLPL	H-2-Kb	20.8	0.060	L2	
20	MSIPYQHV	H-2-Kb	23.9	0.070	M3	
21	VSIRAPRL	H-2-Kb	21.5	0.070	M1	
22	AAFLFKTV	H-2-Kb	25.8	0.080	S2	
23	WSFVYWGL	H-2-Kb	25.6	0.080	L1	Pool #5
24	HSYSSFSKL	H-2-Kb	25.4	0.080	L1	
25	SMFKHHVKL	H-2-Kb	25.2	0.080	L1	
26	STHLWSPL	H-2-Kb	29.1	0.090	L3	
27	MTPMYLQQL	H-2-Kb	30.5	0.090	L3	
28	IMGVFFNGV	H-2-Kb	30.1	0.090	L1	Pool #6
29	ITVNPYYRL	H-2-Kb	32.3	0.100	L2	
30	KIFQAAQL	H-2-Kb	33.0	0.100	L1	
31	ITWDFFLSV	H-2-Kb	33.9	0.100	L1	Pool #7
32	SPNYRFRQSM	H-2-Kb	39.7	0.125	S1	

33	TVVNYVQL	H-2-Kb	39.6	0.125	M2	
34	VSPKYSDLL	H-2-Kb	42.7	0.125	M2	
35	KAFMTLANM	H-2-Kb	41.6	0.125	L3	
36	STRKYFAQTL	H-2-Kb	36.2	0.125	L1	
37	CSAVLFSPL	H-2-Kb	43.6	0.150	L3	
38	VSIRGRWMARL	H-2-Kb	49.7	0.150	L3	Pool #8
39	LSYDLRWTRL	H-2-Kb	49.4	0.150	L2	
40	SDYKFMYM	H-2-Kb	51.5	0.150	L1	
41	IAPMRFVL	H-2-Kb	53.2	0.175	M2	
42	SNQAFYDL	H-2-Kb	61.7	0.175	L2	
43	HFYRYETL	H-2-Kb	52.8	0.175	L2	Pool #9
44	SRLRYLPL	H-2-Kb	62.1	0.175	L2	
45	LMYKYMPIM	H-2-Kb	57.3	0.175	L2	
46	MNYYLLATF	H-2-Kb	66.9	0.200	M2	Pool #10
47	AGWLYNGV	H-2-Kb	70.5	0.200	L3	
48	TWYLAAARM	H-2-Kb	68.4	0.200	L1	

Table S4. List of primers used for RT-qPCR analysis.

Gene	Forward	Reverse
S4Q	5'-CGCTTTTGAAGGTCGTGTATCA-3'	5'-CTGGCTGTGCTGAGATTGTTTT-3'
Ifit-1	5'-CTGGACAAGGTGGAGAAGGT-3'	5'-AGGGTTTTCTGGCTCCACTT-3'
Ifit-2	5'-TGCTCTTGACTGTGAGGAGG-3'	5'-ATCCAGACGGTAGTTCGCAA-3'
Ifit-3	5'-GTGCAACCAGGTCGAACATT-3'	5'- AGGTGACCAGTCGACGAATT-3'
Irf7	5'-GACCGTGTTTACGAGGAACC-3'	5'-GCTGTACAGGAACACGCATC-3'
Isg15	5'-GGAACGAAAGGGGCCACAGCA-3'	5'-CCTCCATGGGCCTTCCCTCGA-3'
Oas1b	5'-AGCATGAGAGACGTTGTGGA-3'	5'-GCGTAGAATTGTTGGTTAGGCT-3'
Ddx58	5'-AAGGCCACAGTTGATCCAAA-3'	5'-TTGGCCAGTTTTCCTTGTCG-3'
Cxcl9	5'-TGGAGTTCGAGGAACCCTAGT-3'	5'-AGGCAGGTTTGATCTCCGTT-3'
Cxcl10	5'-ACGAACTTAACCACCATCT-3'	5'-TAAACTTTAACTACCCATTGATACATA-3'
Mx1	5'-GATGGTCCAAACTGCCTTCG-3'	5'-TTGTAAACCTGGTCCTGGCA-3'
β2M	5'-CTCGGTGACCCTGGTCTTT-3'	5'-CCGTTCTTCAGCATTTGGAT-3'
Mzt2	5'-TCGGTGCCCATATCTCTGTC-3'	5'-CTGCTTCGGGAGTTGCTTTT-3'
Ptp4a2	5'-AGCCCCTGTGGAGATCTCTT-3'	5'-AGCATCACAAACTCGAACCA-3'