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Supplemental Information

Systemically Administered Sindbis Virus in Combination with Immune Checkpoint Blockade Induces Curative Anti-tumor Immunity

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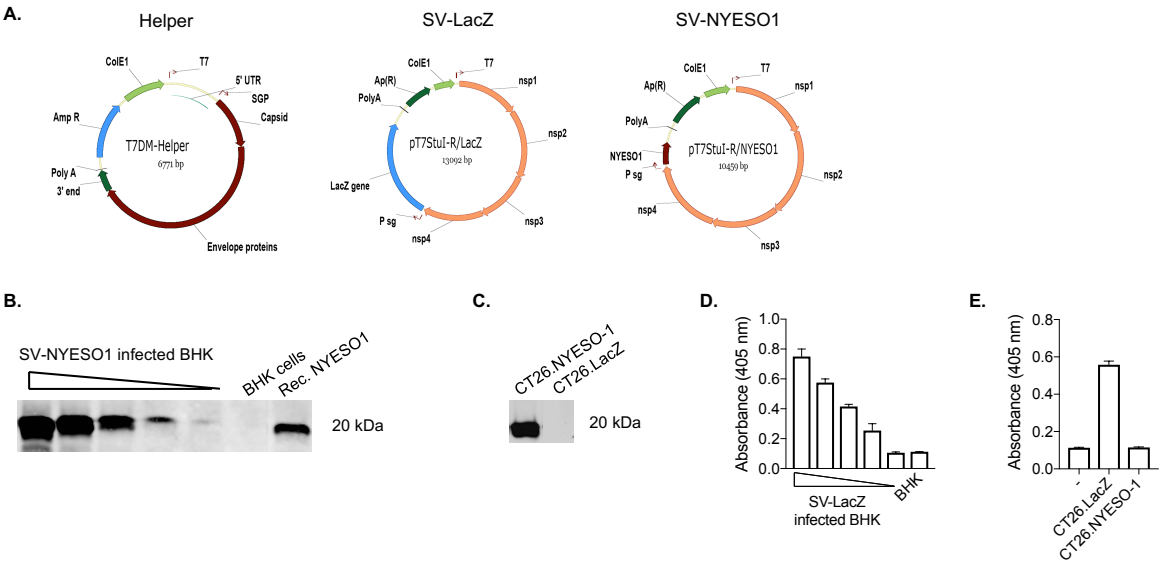
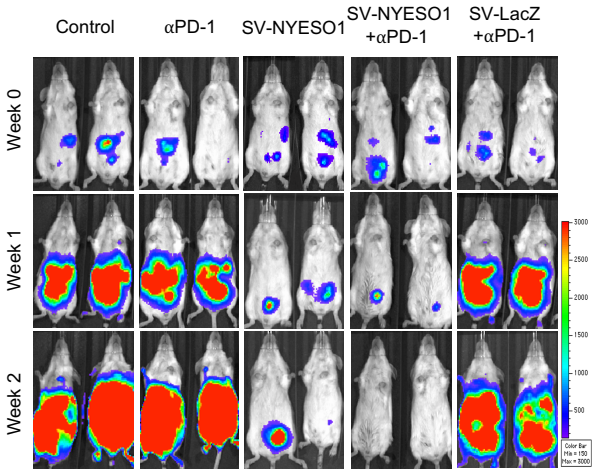
Supplementary Data



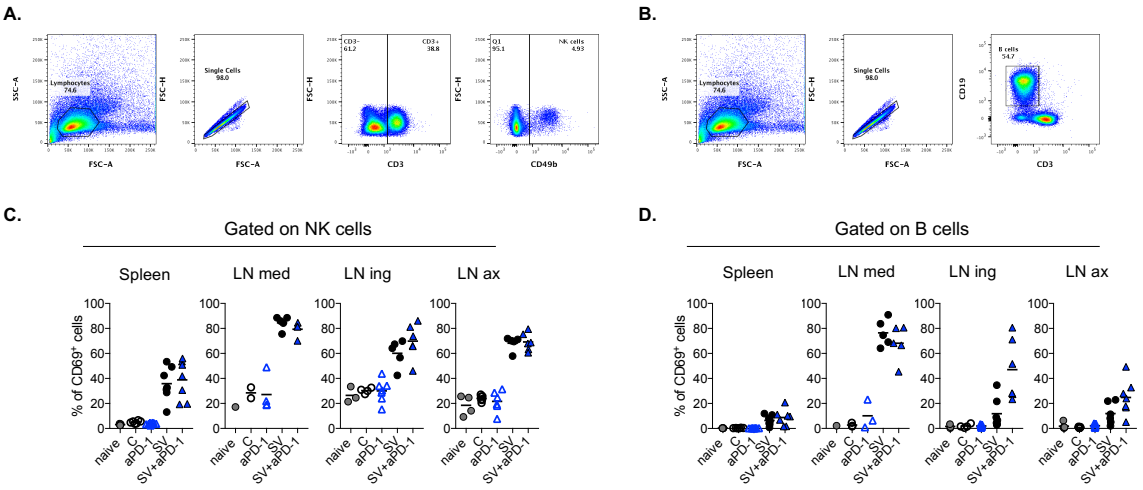
Supplemental Figure S1. Sindbis Virus can be genetically modified to express LacZ or NYESO-1.

A, Map of the helper replicon, SV-LacZ and SV-NYESO-1 plasmids. **B**, To verify NYESO-1 expression of SV-NYESO1, proteins were extracted from SV-NYESO1 infected BHK cells and NYESO-1 expression was detected by Western Blot. As a positive and negative control, recombinant NYESO-1 and uninfected BHK cells were used. **C**, NYESO-1 expression in CT26.Fluc.NYESO1 was verified by Western blot. CT26.Fluc.LacZ was used as a negative control **D**, To verify LacZ expression of SV-LacZ, Protein were extracted from SV-LacZ infected BHK cells and LacZ expression was detected using the mammalian β-Galactosidase assay kit. As a negative control, uninfected BHK cells were used. **E**, LacZ expression of the mouse colon carcinoma cell line CT26.Fluc.LacZ was verified using the mammalian β-Galactosidase assay kit. As a negative control, CT26.Fluc.NYESO1 cells were used.



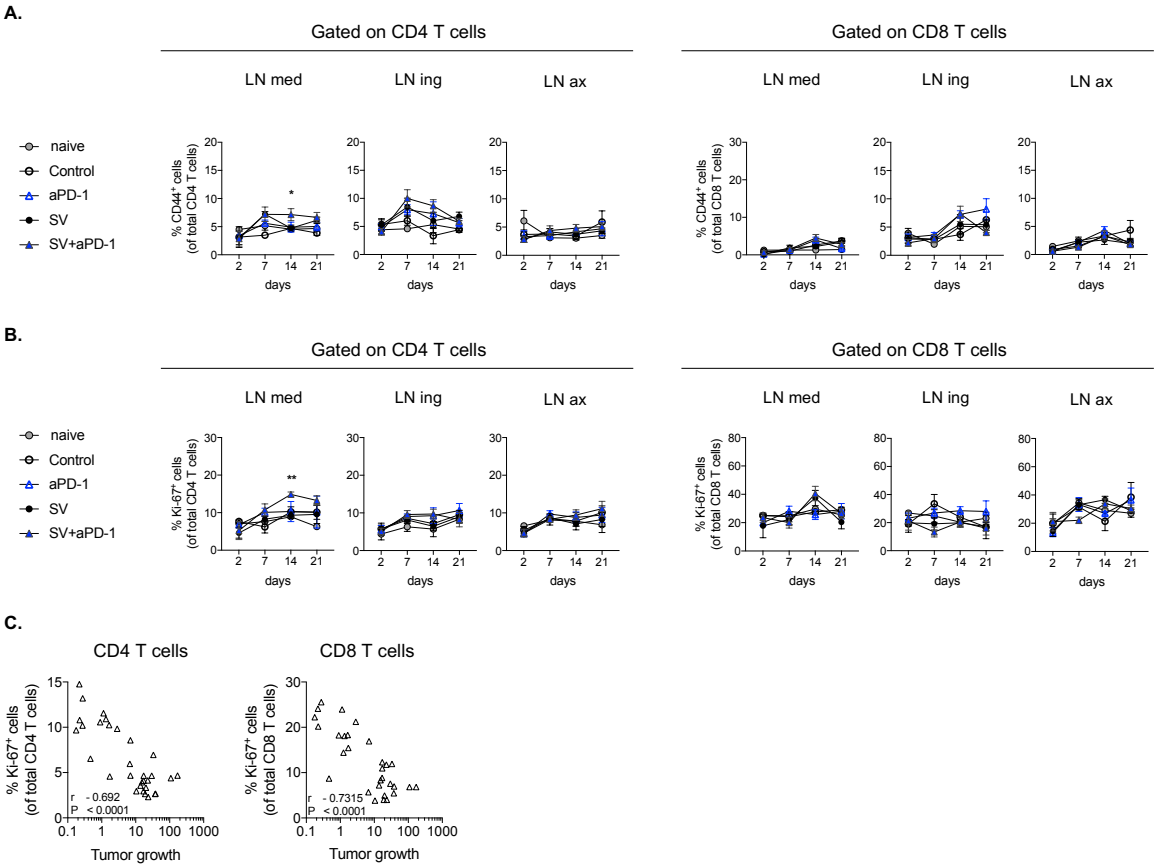
Supplemental Figure S2. Noninvasive bioluminescence images of CT26.Fluc.NYESO1 tumor bearing mice during SV treatment.

Representative bioluminescence images of three independent experiments showing control and treated mice bearing CT26.Fluc.NYESO1 tumors. Images were taken one day before starting SV treatment (day 0) and at weeks 1 and 2.



Supplemental Figure S3. SV-NYESO1 induces an early and systemic activation of NK and B cells.

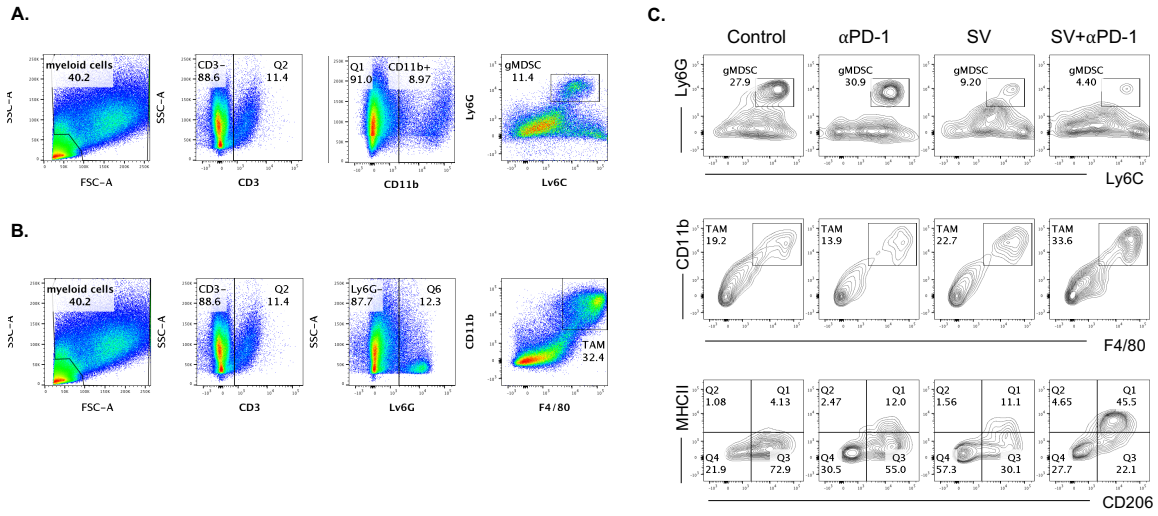
CT26.Fluc.NYESO1 tumor cells were injected into BALB/c mice on day -4. One injection of anti-PD-1 (250 µg) and/or SV-NYESO1 was given to the respective groups on day 0 and 1, respectively. On day 2, mice were sacrificed and organs were removed and prepared for flow cytometry analysis. **A** and **B**, Flow cytometry gating strategy to define NK cells (A) and B cells (B). **C** and **D**, Percentage of CD69 expression by NK cells (C) and B cells (D). Left to right: spleen, mediastinal (LN med), inguinal (LN ing) and axillary lymph nodes (LN ax). Results are representatives from two independent experiments. Lines represent means and statistical significance was determined with the Kurskal-Wallis test followed by the Dunns' test.



Supplemental Figure S4. T cell activation in peripheral lymphoid organs over the course of SV-NYESO1 treatment in presence or absence of anti-PD-1.

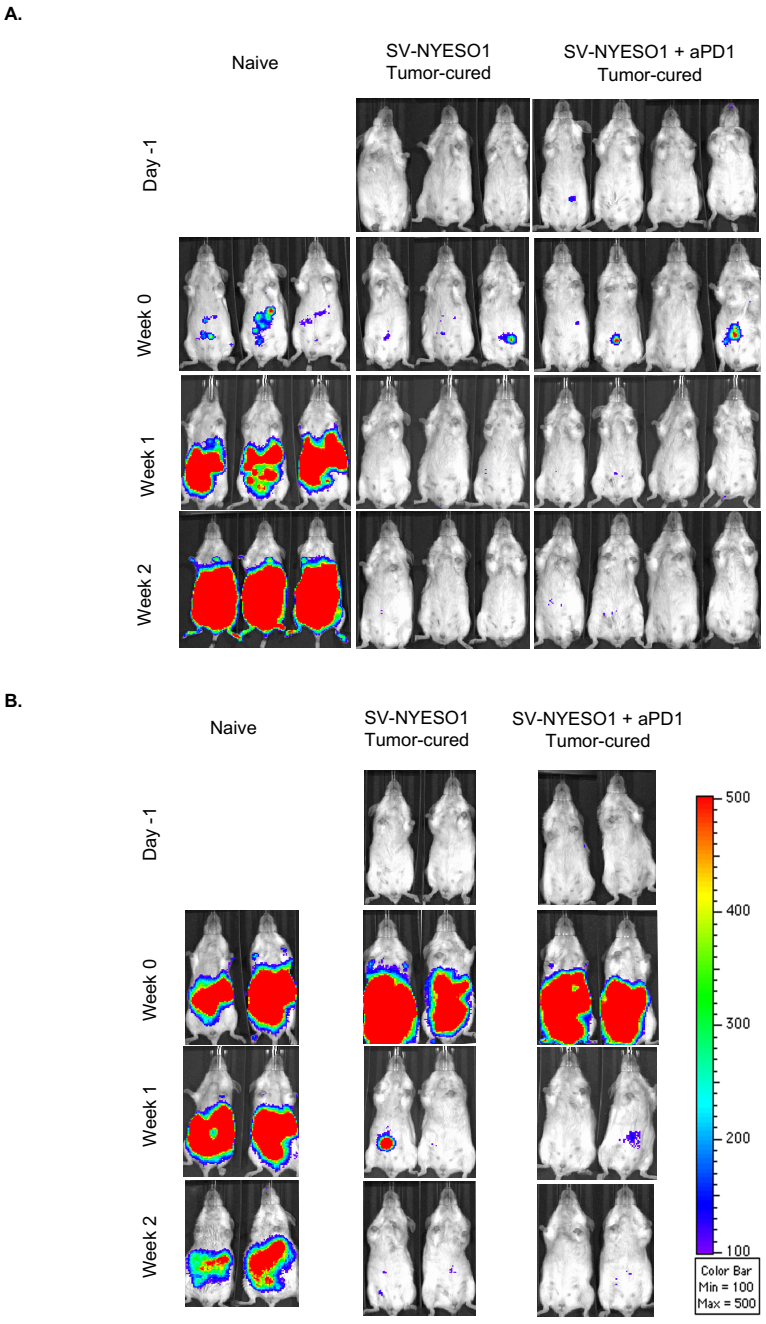
Tumor bearing BALB/c mice were left untreated or were treated with SV with or without anti-PD-1. Mice were sacrificed on day 2, 7, 14 or 21 to analyze the T cell immune response in spleen and mediastinal (med), inguinal (ing) and axillary (ax) lymph nodes (LN). **A** and **B**, Percentage of CD44 (A) and Ki-67 (B) expression by $CD4^+$ T cells and $CD8^+$ T cells in naive mice, as well as, control or treated tumor bearing mice using flow cytometry (n=8 mice per group). Left graphs: $CD4^+$ T cells. Right graphs: $CD8^+$ T cells. Symbols summarizing data from two independent experiments. Statistical significance between groups treated with SV in presence or absence of anti-PD-1 was determined with the Mann-Whitney test. **C**, Correlation of splenic

CD4$^+$ T cells or CD8$^+$ T cells Ki-67 expression against tumor growth on day 14 by the Spearman-rank correlation test. Results are representatives from two independent experiments. n.s > 0.05, $*P < 0.05$, $**P \leq 0.01$.



Supplemental Figure S5. Flow cytometry gating strategy for granulocytic-myeloid derived supressor cell and Tumor-associated macrophages in tumor.

A, Gating strategy of granulocytic-myeloid derived suppressor cells (gMDSC) by flow cytometry. **B**, Gating strategy of tumor-associated macrophages by flow cytometry. **C**, Representative flow cytometry plots of the frequencies of gMDSCs, TAMs and macrophage type 2 like cells from indicated groups.



Supplemental Figure S6. Whole body bioluminescence images of rechallenged tumor cured mice.

Tumor cured mice were injected i.p. with 7 x 10^4 CT26.Fluc.NYESO1 cells (A; n=7) or 5 x 10^4 CT26.Fluc.LacZ cells (B; n=4) at 200 days after SV-NYESO1 or SV-NYESO1 with anti-PD-1

treatments. Bioluminescence was recorded one day before re-challenge for tumor cured mice as background signal control; one day after cells inoculation (Week 0) and then weekly. The scale used to prepare both figures is shown in (B).

Supplemental Table S1. Primers and conditions for RT-PCR used to titer vectors

cDNA (ThermoScript ™ RNaseH-reverse transcriptase (Invitrogen)		
primer	**Sequence**	**cDNA Cycle**
cDNA5R	5'- TTTTTGAAATGTTAAAAACAAAATTTTGTTG	2 hours at 60 °C
QPCR (IQ SYBR Green Supermix (BioRad))		
primer	**Sequence**	**cDNA Cycle**
7692F	5'-TGATCCGACCAGCAAAACTC	5 min at 95 °C 40 x [95 °C 20 sec; 60 °C 30 sec; 72 °C 30 sec]
cDNA5R	5'- TTTTTGAAATGTTAAAAACAAAATTTTGTTG	

Supplemental Table S2. FACS panel for surface markers.

Antibody	Clone	Fluorochrome	Vendor
CD3	17A2	BV786	Biolegend
CD3	17A2	BV605	Biolegend
CD4	RM4-4	PerCP-Cy5.5	Biolegend
CD8	53-6.7	APC-H7	BD Bioscience
CD11b	M1/70	BV786	Biolegend
CD11c	N418	PercP-Cy5.5	Biolegend
CD19	6D5	PE-Cy7	Biolegend
CD44	IM7	BV605	Biolegend
CD49b	DX5	PE	Biolegend
CD62L	MEL-14	Alexa Fluor 700	Biolegend
CD69	H1.2F3	FITC	Biolegend
Ly6C	HK1.4	PE-Cy7	Biolegend
Ly6G	1A8	BV421	Biolegend
PD-1	29F.1A12	APC	Biolegend
PD-L1	10F.9G2	PE	Biolegend
F4/80	T45-2342	PE-CF594	BD Bioscience
IA/IB	M5/114.15.2	V500	BD Bioscience

Supplemental Table S3. FACS panel for intracellular staining.

Antibody	Clone	Fluorochrome	Vendor
FoxP3	259D/C7	PE-CF594	BD Bioscience
Ki-67	16A8	BV421	Biolegend