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Supplemental Information

Self-Delivering RNAi Targeting PD-1 Improves Tumor-Specific T Cell Functionality for Adoptive Cell Therapy of Malignant Melanoma

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Table S1. List of PDCD1 (NM_005018) targeting sequences selected for screening.

##	ID	Target sequence
1	PD60	TATTATATTATAATTATAAT
2	PD61	TCTATTATATTATAATTATA
3	PD62	CATTCCTGAAATTATTTAAA
4	PD63	CTATTATATTATAATTATAA
5	PD64	AGTTTCAGGGAAGGTCAGAA
6	PD65	TGTGGTTCTATTATATTATA
7	PD66	TGTGTTCTCTGTGGACTATG
8	PD67	CCCATTCCTGAAATTATTTA
9	PD68	TGCCACCATTGTCTTTCCTA
10	PD69	AAGTTTCAGGGAAGGTCAGA
11	PD70	CTGTGGTTCTATTATATTAT
12	PD71	TTCTATTATATTATAATTAT
13	PD72	TTTCAGGGAAGGTCAGAAGA
14	PD73	CTTGGAACCCATTCCTGAAA
15	PD74	TCCCTGTGGTTCTATTATAT
16	PD75	CCTGTGGTTCTATTATATTA
17	PD76	TGGAACCCATTCCTGAAATT
18	PD77	CCTTCCCTGTGGTTCTATTA
19	PD78	TTCCCTGTGGTTCTATTATA
20	PD79	CACAGGACTCATGTCTCAAT

Table S2. Antibodies used in flow cytometry

Marker	Conjugate	Clone
CCR5[2]	FITC	HEK/1/85a
CCR7[1]	PE	150503
CD107a[2]	FITC	H4A3
CD3[2]	PE-Cy7	UCHT1
CD3[6]	-	SP7
CD4[3]	PE-CF594	S3.5
CD8[2]	APC-Cy7	SK1
CD28[2]	PerCP-Cy5.5	CD28.2
CD45RA[2]	APC	HI100
CD69[2]	APC-Cy7	FN50
CD95[4]	APC	NOK-1
CXCR3[2]	PB	G025H7
IFN-g[2]	PE-Cy7	4s.B3
MICA/B[2]	APC	6D4
TNF-a[2]	APC	MAb11
PD1[2]	PB	EH12.2H7
PDL1[1]	PE-Cy7	MIH1
PDL2[1]	PE-CF594	MIH18
TRAIL-R2[2]	PE	DJR2-4 (7-8)
ULBP2/5/6	PerCP	165903
Goat Anti-Rabbit IgG H&L	Alf488	-

[1]BD Biosciences (San Jose, CA), [2]BioLegend (San Diego, CA), [3]Invitrogen (Carlsbad, CA), [4]Miltenyi Biotech, [5]eBioscience (San Diego, CA).

Figure S1

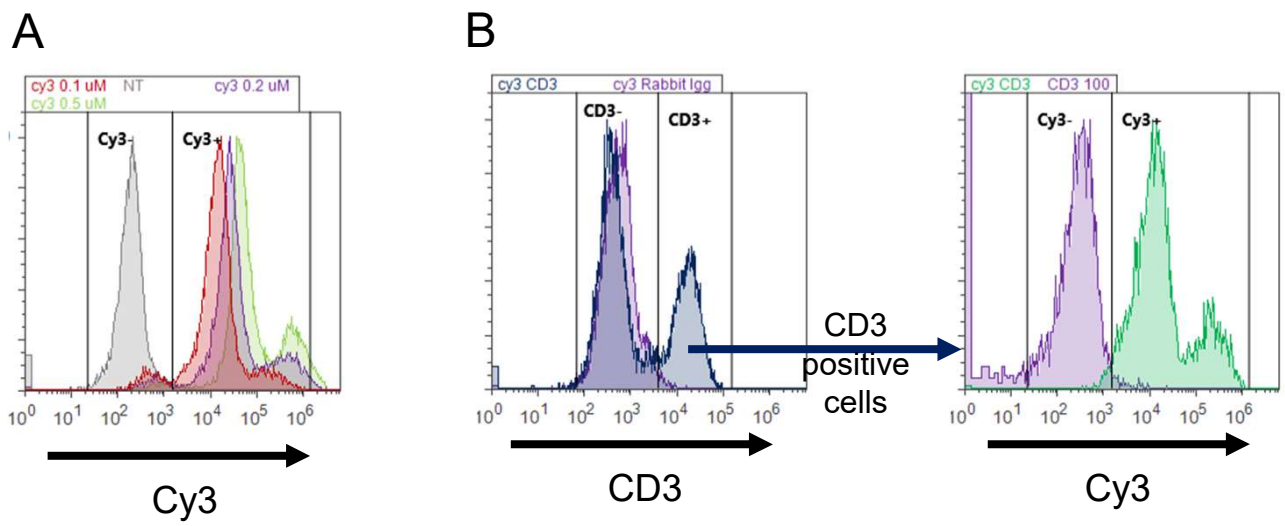


Figure S1.
Uptake of sdRNA by CD3 positive PBMC cells (T cells).
Peripheral blood mononuclear cells cultured in presence of PHA (1.5% final; Gibco, 10576015) and IL2 (1000 U/ml; PHC0026) were incubated with 0.1 µM, 0.2 µM or 0.5 µM MAP4K4-cy3 sdRNA for 72 h. Cells treated with 0.1 µM sdRNA were stained with rabbit anti-CD3 antibody (see antibody table) at 1:100 dilution or with rabbit IgG, as a reference, followed by 1:2000 diluted secondary Alexa Fluor-488 antibody. Fluorescence quantitative data was acquired with Attune Flow Cytometer (Applied Biosystems) and analyzed using FlowJo software (Treestar Inc).
Overall transfection efficiency of cy3-conjugated sdRNA was gated at 92.8%, 95.1% and 97% cells incubated with 0.1 µM, 0.2 µM or 0.5 µM sdRNA, respectively (A). Out of 35% of singlet cells gated positive for CD3, 97.4% show positive sdRNA uptake (B).

Figure S2

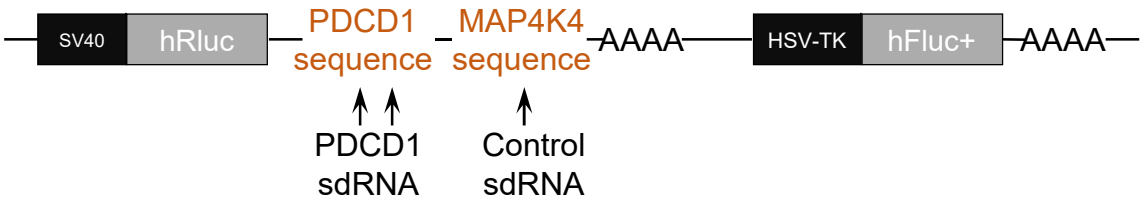


Figure S2.
Schematic representation of a dual-luciferase reporter plasmid constructed for sdRNA screening.
PD1 sdRNA target sequence was inserted downstream of Renilla under the same promoter into psiCheck2 vector (Promega, C8021). Positive control targeting sequence for MAP4K4 sdRNA was cloned into the same cassette to monitor for cross-assay variability. Renilla luciferase activity was normalized to firefly luciferase activity expressed under a separate promoter.

Figure S3

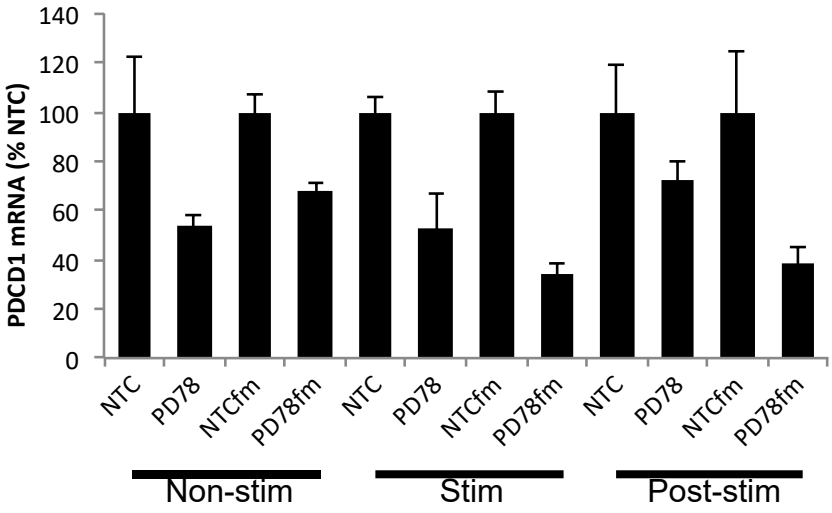


Figure S3.
Increased efficacy of fully modified PD78fm sdRNA in activated T cells.
Primary T cells were activated with anti-CD3/CD28 magnetic beads for 5 days or left unstimulated ("Non-stim") in a growth medium - AIM-V supplemented with 10% FBS and 500 U/ml IL2 (ProSpec). Activated cells were transfected with sdRNA either in presence of the beads ("Stim"), or after beads removal on a Dynamagnet ("Post-stim"). For transfection, all three groups of cells were incubated with 2 µM PD78 or PD78fm sdRNA for 72 h. Gene expression was detected using Taqman probes in one-step qPCR, normalized for GAPDH expression and adjusted for the standard curve. Data plotted as percent of non-treated cells (n = 3; mean ± SD).

Figure S4

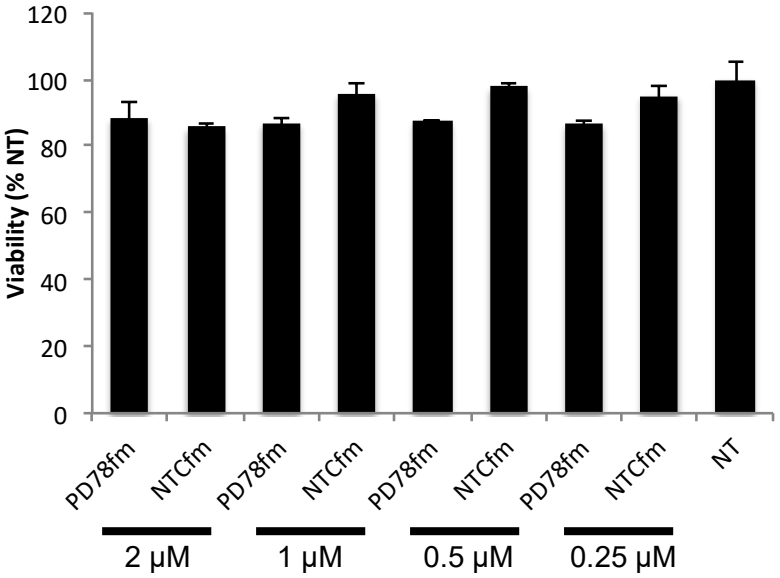


Figure S4.
Viability of T cells treated with PD78fm sdRNA.
T cells were activated with anti-CD3/CD28 magnetic beads and incubated with indicated doses of PD78fm sdRNA for 72 h in the presence of beads. Viability was determined using CellTiter-Blue reagent. Fluorescence of converted dye was measured in SpectraMax plate reader (Molecular Devices) and expressed as percent of non-treated (NT) cells (n = 3; mean ± SD). NTC (non-targeting control) serves as non-specific reference of sdRNA effect on cells.

Figure S5

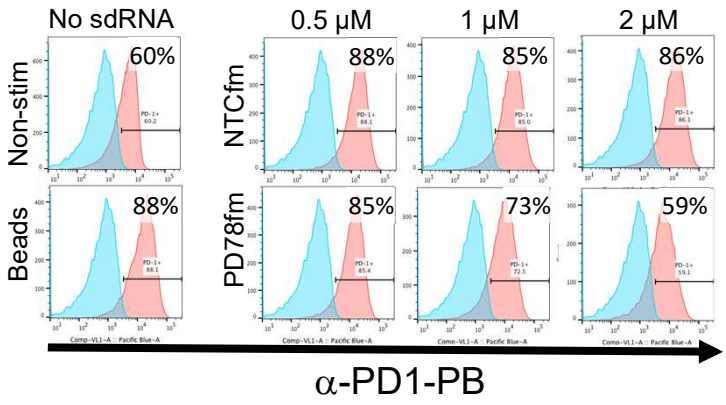
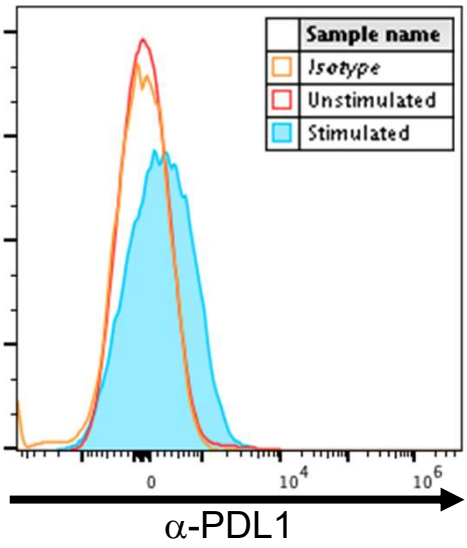


Figure S5
Intracellular PD-1 levels after treatment with increasing concentrations of PD78fm or the control sdRNA NTCfm.
T cells were cultured for four days in the presence of PD78fm at varying concentrations or non-targeting control (NTCfm) followed by stimulation with CD3/CD28 beads

Figure S6

A



B

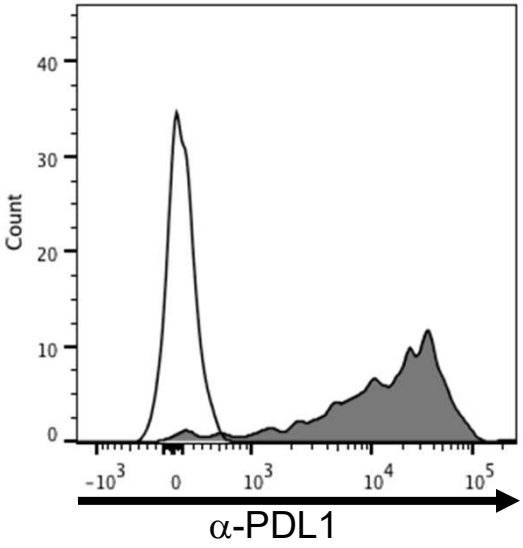


Figure S6

Surface PD-L1 levels

A. T cells were stimulated overnight with CD3/CD28 beads and stained for PD-L1
B. Short term passage melanoma cell line KADA PD-L1 surface expression

Figure S7

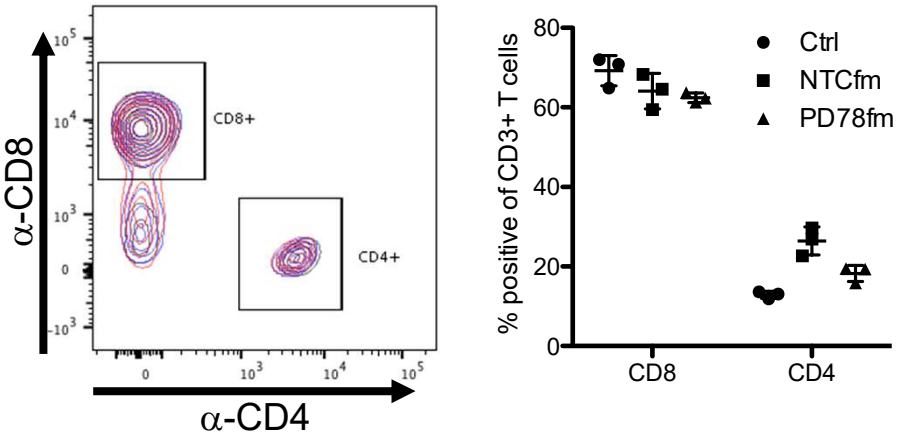


Figure S7
CD4⁺ and CD8⁺ cell frequencies in TIL after REP
T cells were expanded for two weeks in CellGro medium supplemented with 2% human AB serum, 30 ng/ml OKT-3 and 300 U/ml IL-2. sdRNA was added at a 2 µM concentration on days 4, 8 and 12