frontiers

Supplementary Material

TIM-3, LAG-3, or 2B4 gene disruptions increase the anti-tumor response of engineered T cells

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1 Supplementary tables

Table 1: List monoclonal antibodies

Specificity	Antibody	Color	Clone	Catalogue	Source	Experiment
anti Human	2B4 (CD244)	PE-dazzle	C1,7	329522	BioLegend	C/E/I
anti Human	7AAD			420404	BioLegend	E/L
anti Human	Annexin V	Alexa fluor 647		640912	BioLegend	L
anti Human	CD107a (LAMP-1)	PE eFluor610	eBioH4A3	61-1079-42	Invitrogen	C/E
anti Human	CD138	PE	DL-101	352306	BioLegend	I
anti Human	CD138	PE/Cy5	B-A38	A54191	Beckman Coulter	L
anti Human	CD160	PE/Cy7	BY55	341212	BioLegend	E
anti Human	CD25	APC/Cy7	BC96	302614	BioLegend	C/E
anti Human	CD3	BV510	OKT3	317332	BioLegend	C/E/I
anti Human	CD3	FITC	SK7	11-0036-42	Invitrogen	C/E
anti Human	CD3	PE/Cy7	OKT3	25-0037-42	Invitrogen	C/E
anti Human	CD3	BUV661	SK7	741692	BD Biosciences	C/E
anti Human	CD3	PerCP	UCHT1	MA1-10181	Invitrogen	P
anti Human	CD38	PE/Cy7	HB-7	356608	BioLegend	I/L
anti Human	CD39	BUV737	TU66	58781	BD Biosciences	E
anti Human	CD4	APC/Cy7	OKT4	317418	BioLegend	C/E
anti Human	CD4	APC	OKT4	17-0048-42	Invitrogen	C/E
anti Human	CD4	PE/Cy7	OKT4	25-0048-42	Invitrogen	E

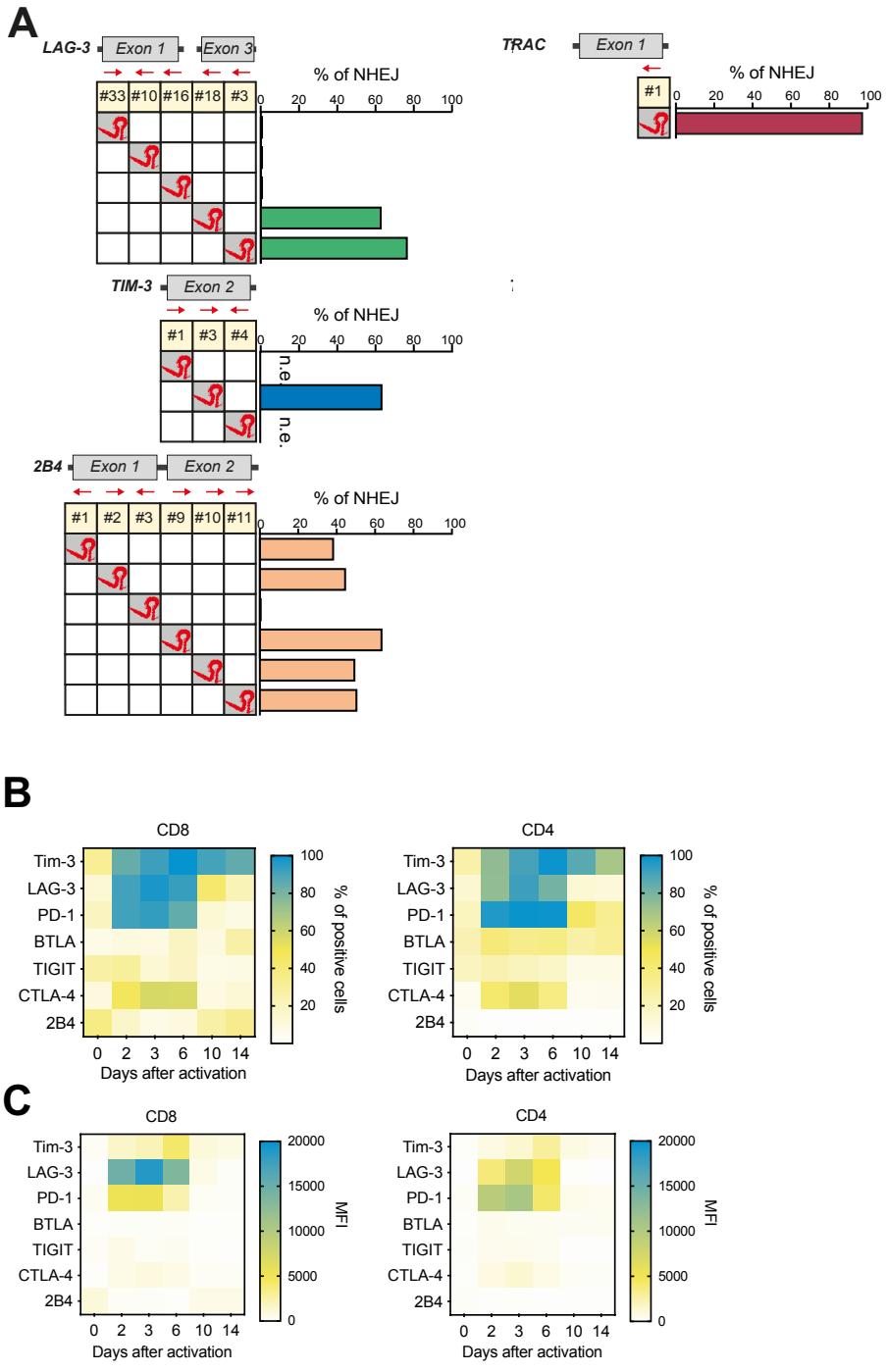
anti Human	CD40L (CD154)	PE	TRAP1	555700	BD Biosciences	C/E
anti Human	CD45RA	BUV496	H100	750258	BD Biosciences	C/E
anti Human	CD45RA	PE	H100	12-0458-42	Invitrogen	E
anti Human	CD48	APC Vio770	REA426	130-106-470	Miltenyi	I/L
anti Human	CD62L	BUV737	DREG-56	741843	BD Biosciences	C/E
anti Human	CD62L	Pacific Blue	DREG-56	304826	BioLegend	E
anti Human	CEACAM (CD66a/c/e)	PE	ASL-32	342304	BioLegend	L
anti Human	CD69	APC	FN50	310910	BioLegend	C/E
anti Human	CD8	BV785	SK1	344740	BioLegend	C/E
anti Human	CD8	PerCP	SK1	345774	BD	C/E
anti Human	CD8	APC/Cy7	SK1	344714	BioLegend	C/E
anti Human	CD8	BUV395	RPA-T8	9240259	BD Biosciences	E
anti Human	CD80	PE/Cy7	2D10	305218	BioLegend	I
anti Human	CD95 (FAS)	FITC	DX2	305606	BioLegend	E
anti Human	CTLA-4 (CD152)	BV605	BNI3	369610	BioLegend	C/E/I
anti Human	DAPI			D9542	Sigma-aldrich	C/E/I
anti Human	ERK1/2 (pT202/pY204)		20A	562981	BD Biosciences	P
anti Human	Galectin-9	APC	9M1-3	348908	BioLegend	L
anti Human	hCD45	APC/Cy7	H130	304014	BioLegend	I
anti Human	HLA-A2	PE	BB7.2	12-9876-42	Invitrogen	I
anti Human	HLA-A2	APC	BB7.2	343308	BioLegend	L
anti Human	HLA-DR	BV480	G46-6	566113	BD Biosciences	C/E/I
anti Human	HLA-DR	PE	L243	307606	BioLegend	C/E
anti Human	HLA-DR	APC/Cy7	L243	307618	BioLegend	E

anti Human	IFNg	APC/Cy7	4S.B3	502530	BioLegend	C/E
anti Human	IL2	APC	MQ1-17H12	500310	BioLegend	C/E
anti Human	Ki-67	Alexa fluor 488	Ki-67	350508	BioLegend	C/E
anti Human	KLRG1 (MAFA)	BV785	2F1/KLRG1	138429	BioLegend	E
anti Human	LAG3 (CD223)	APC	REA351	130-119-567	Miltenyi	C/E/I
anti Mouse	mCD45	PerCP	30-F11	103130	BioLegend	I
anti Human	NGFR (CD271)	APC	ME20.4	17-9400-42	Invitrogen	L
anti Human	PD-1 (CD279)	BV650	EH12-2H7	329950	BioLegend	C/E/I
anti Human	PD-1 (CD279)	PE/Cy7	EH12.2H7	329918	BioLegend	C/E
anti Human	PD-1 (CD279)	APC	EH12.2H7	329908	BioLegend	E
anti Human	PD-1 (CD279)	BV421	EH12-2H7	329920	BioLegend	C/E
anti Human	TIGIT	PE/Cy7	MBSA43	25-9500-42	Invitrogen	C/E
anti Human	TIGIT	BB700	741182	747846	BD Biosciences	E
anti Human	TIM3 (CD3663)	PE	REA635	130-119-785	Miltenyi	C/E/I
anti Human	TNFa	PE/Cy7	MAb11	502930	BioLegend	C/E
anti Human	Vβ13.1	FITC		IM1554	Beckman Coulter	E
	Zombie aqua	BV510		423102	BioLegend	L
	Zombie violet	Pacific blue		423114	BioLegend	C/E/I

Legend: C= chronic; E= editing; I= in vivo; L= cell lines; P= phosphoFlow

2 Supplementary Figures

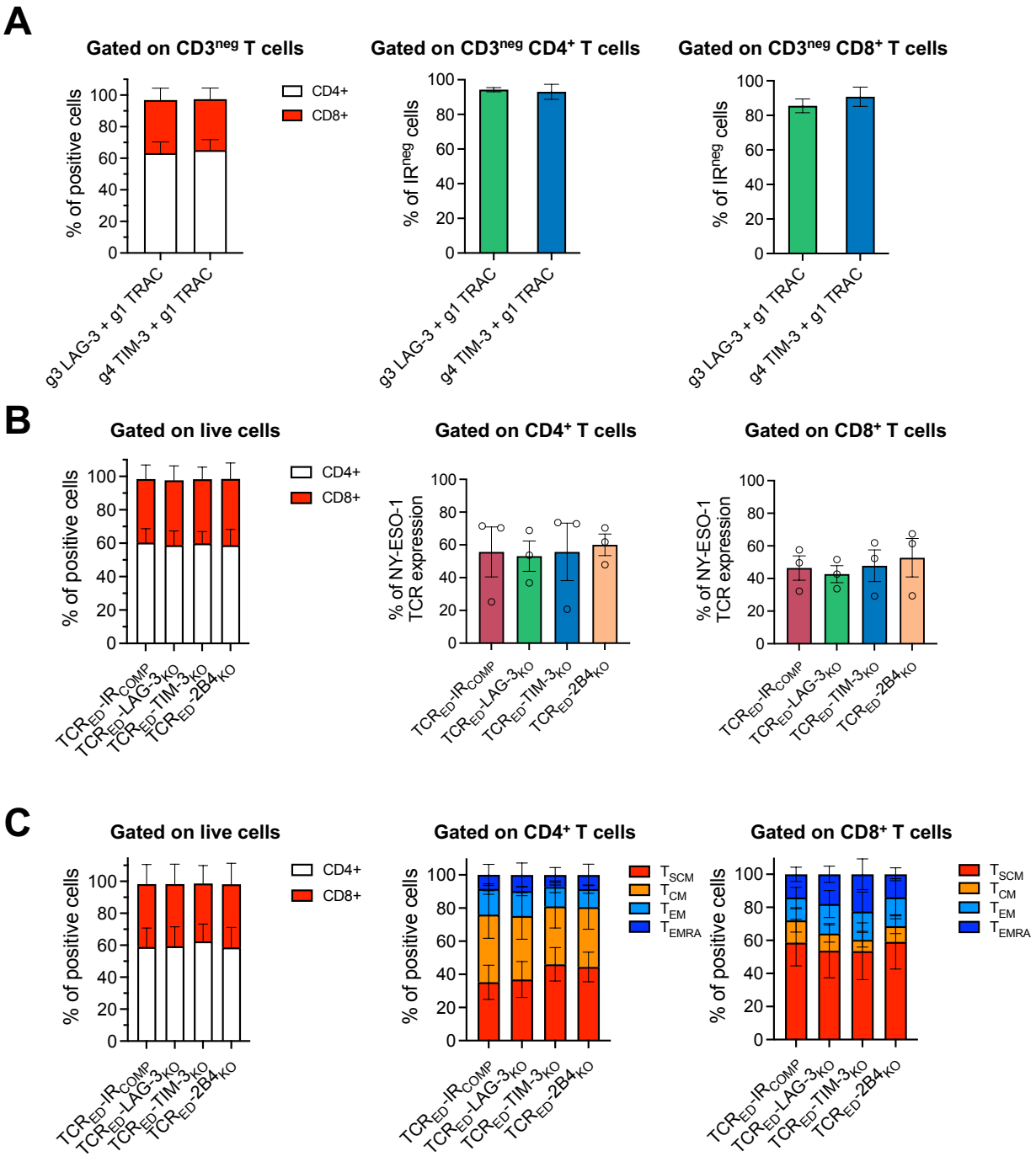
Supplementary Figure 1



Supplementary Figure 1. Design and screening of multiple gRNAs targeting the endogenous TCR or inhibitory genes.

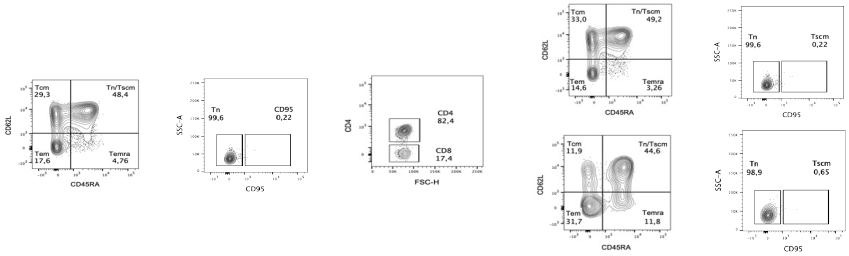
(**A**) Screening of gRNAs targeting the coding regions of *LAG-3*, *TIM-3*, *2B4*, and *TRAC* genes. For each gRNAs, the frequency of NHEJ events is shown. Each red arrow indicates a single gRNA and the direction of the arrows indicate the genomic orientation of the gRNA sequences. Heatmap showing the frequencies (**B**) and the mean fluorescence intensity-MFI (**C**) of IR^+ $CD8^+$ (left) and $CD4^+$ (right) T cells in resting conditions (day 0) and in the first 14 days after polyclonal stimulation. The percentage of positive cells and the MFI is indicated by a color gradient ranging from white (0%) to blue (100%), as indicated in the right panel. Data from a single healthy donor were used for gRNA screening (A). In panel B and C, data from 3 different biological replicates are shown (N=3).

Supplementary Figure 2

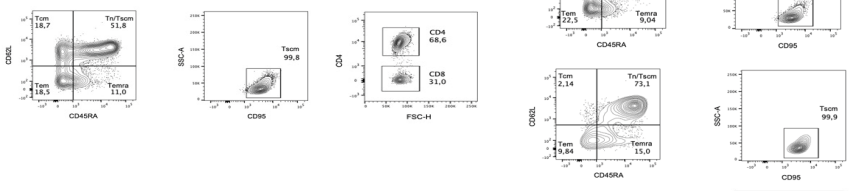


D

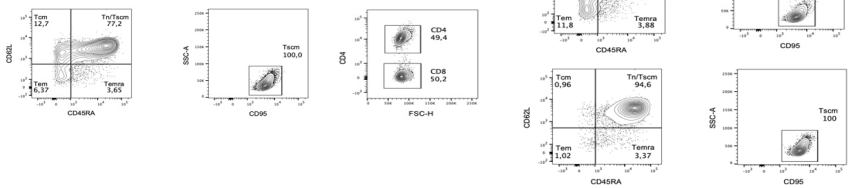
PBMC



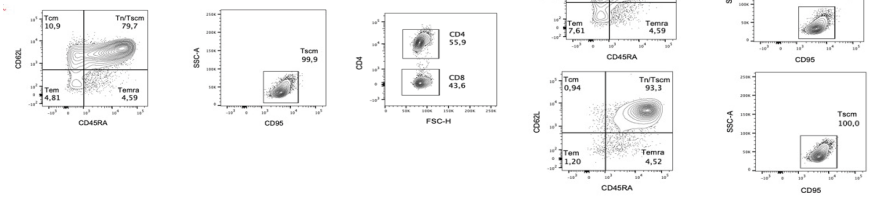
UT



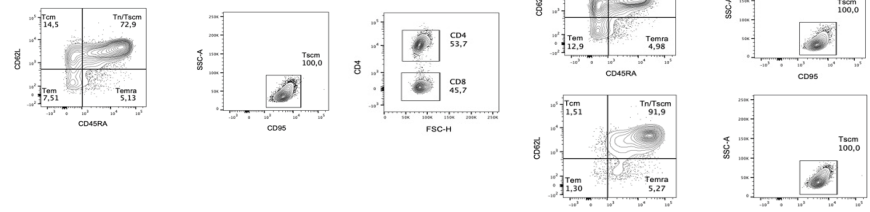
TCR_{ED}-IR_{COMP}



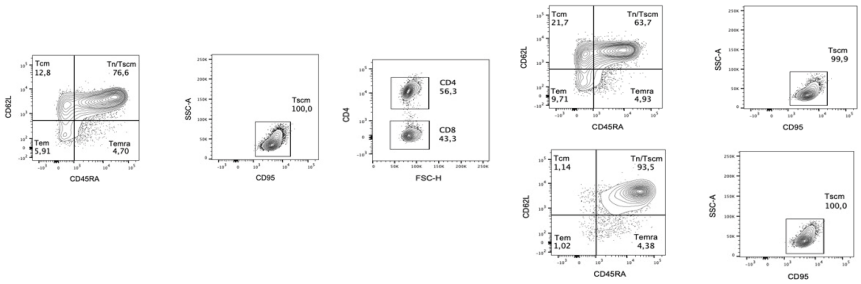
TCR_{ED}-LAG-3_{KO}



TCR_{ED}-Tim-3_{KO}



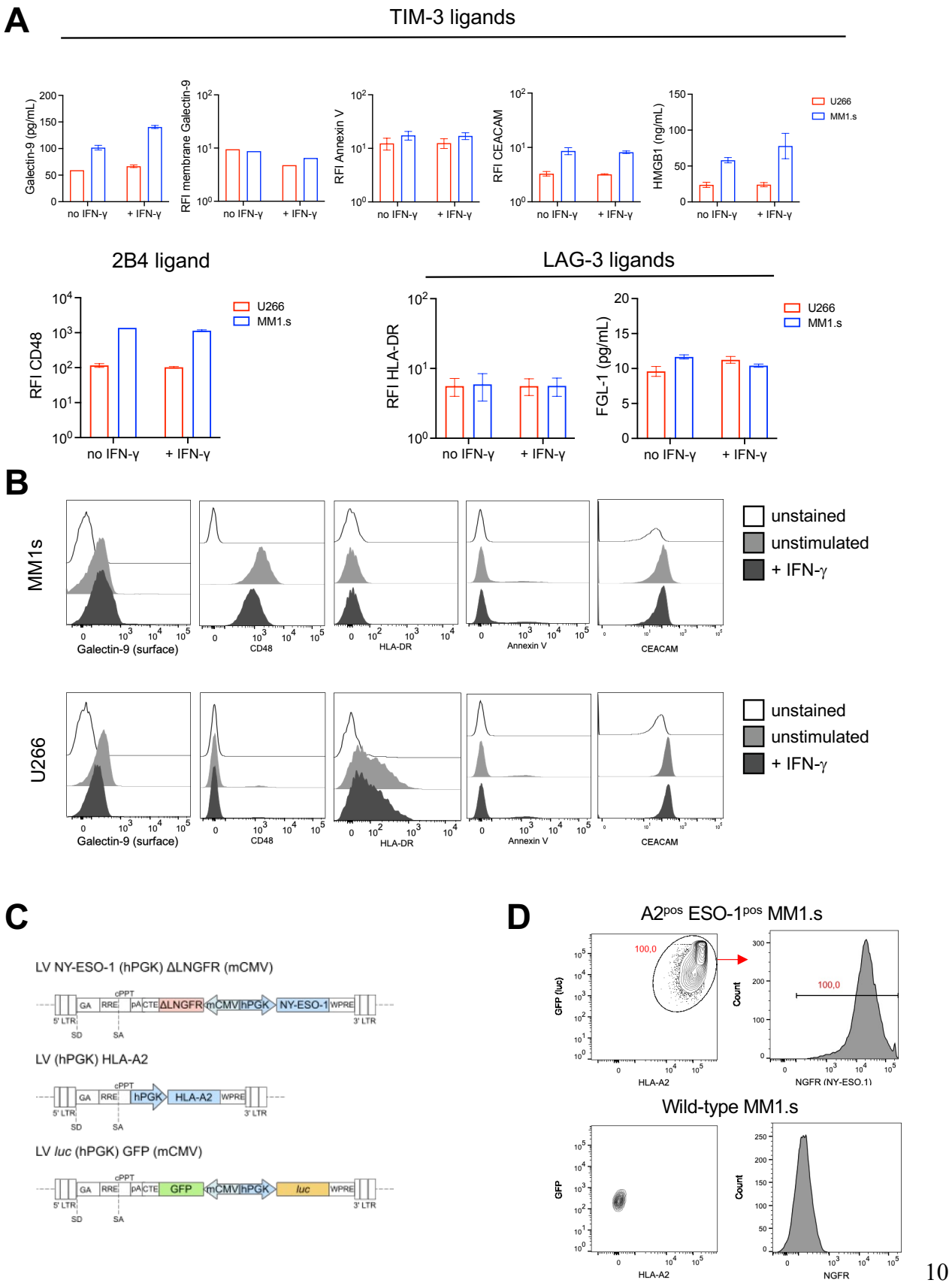
TCR_{ED}-2B4$_{KO}$



Supplementary Figure 2. Gene modifications and memory compositions of $CD4^+$ and $CD8^+$ TCR_{ED}-IR_{KO} T cells.

(A) Bar graphs showing the frequencies of $CD4^+$ and $CD8^+$ T cells (left panel), the frequencies of $LAG3^{neg}$ (green bars) and TIM-3^{neg} (blue bars) $CD4^+$ and $CD8^+$ T cells (central and right panels, respectively) in $CD3^{neg}$ T cells 5 days after CRISPR/Cas9 delivery (day 7 of the manufacturing protocol) in the indicated cellular products (N=3). (**B**) Bar graphs showing the frequencies of $CD4^+$ and $CD8^+$ T cells (left panel) and the frequencies of $CD4^+$ and $CD8^+$ cells (central and right panels, respectively) expressing the NY-ESO-1-specific TCR at day 10 of the manufacturing protocol in the indicated cellular products (N=3). (**C**) Bar graphs showing the frequency of $CD4^+$ and $CD8^+$ T cells (N=3) and the memory phenotype of $CD4^+$ and $CD8^+$ T cells (N=4) (central and right panel, respectively) at the end of the manufacturing protocol (day 18) in all tested cellular products. Data are shown as mean ± SEM from 3-4 different biological replicates. (**D**) Representative dot plots depicting the frequencies of effector and memory $CD3^+$, $CD4^+$, $CD8^+$ T cell subsets in TCR_{ED}-IR_{COMP} and TCR_{ED}-IR_{KO} cells.

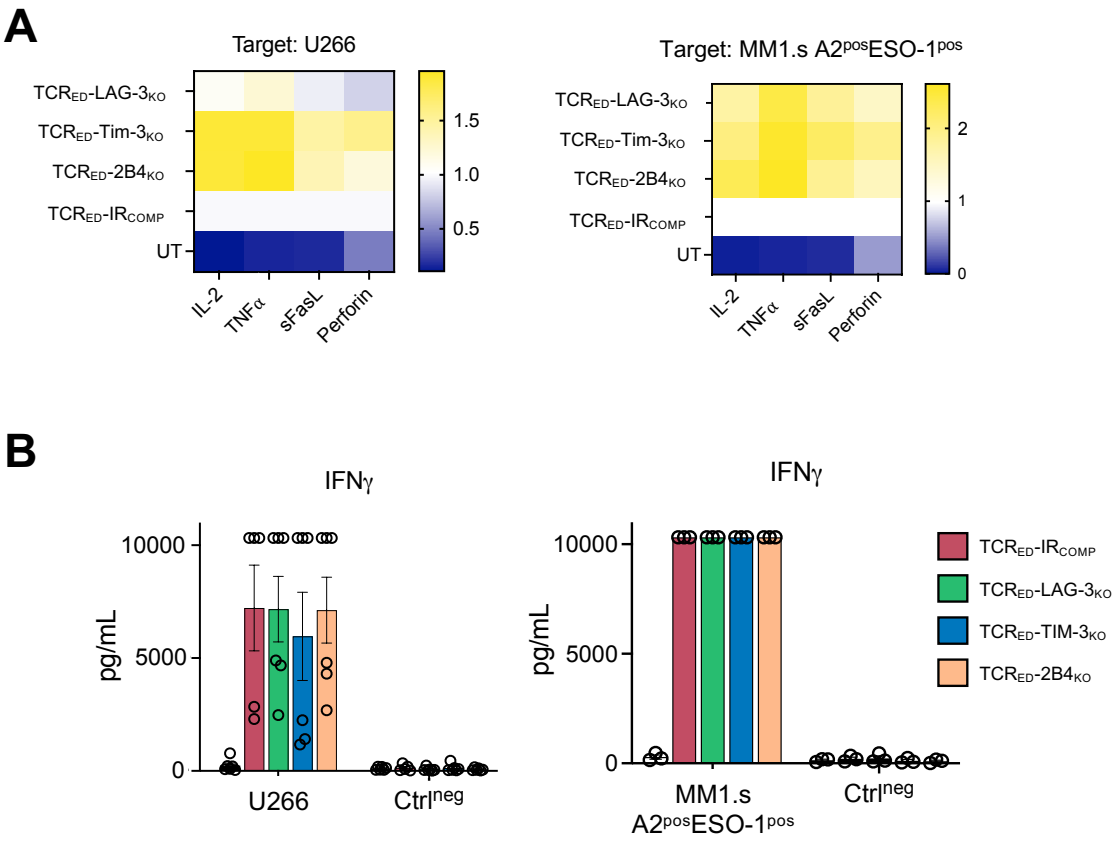
Supplementary Figure 3



Supplementary Figure 3. Generation and characterization of HLA-A2^{POS} NY-ESO-1^{POS} human tumor cell lines.

(A) Quantification in U266 or MM1.s in resting conditions or upon 48 hours IFNγ stimulation of soluble and membrane bound galectin-9 (N=2/3), Annexin V (N=6), CEACAM (N=3) and HMGB1 (N=2/3) (ligands of TIM-3; upper panels); CD48 (ligand of 2B4; bottom left; N=5) and HLA-DR (N=6) and FGL-1 (N=2/3) (ligands of LAG-3; bottom right). (**B**) Representative histograms showing membrane bound Galectin-9 (Galectin 9 – surface), CD48, HLA-DR, Annexin V and CEACAM expression in MM1.s and U266 unstimulated (light grey histograms) and 48h after IFNγ stimulation (dark grey histograms). Unstained cells (white histograms) are shown as control. (**C**) Schematic representations of lentiviral vectors encoding for the NY-ESO-1 antigen under hPGK promoter and ΔLNGFR selection marker under mCMV promoter [LV NY-ESO-1 (hPGK) ΔLNGFR (mCMV), upper panel] or encoding for HLA-A2 under hPGK promoter [LV (hPGK) HLA-A2), middle panel] or encoding for the firefly luciferase under hPGK promoter and green fluorescent protein under mCMV promoter [LV luc (hPGK) GFP (mCMV), lower panel]. (**D**) Representative plots of MM1.s engineered to express luciferase, HLA-A2 and NY-ESO-1 (upper panel) or MM1.s wt cells (bottom panel). In panel A, mean ± SEM from 2-6 technical replicates are shown, with the exception of membrane Galectin-9 (single replicate).

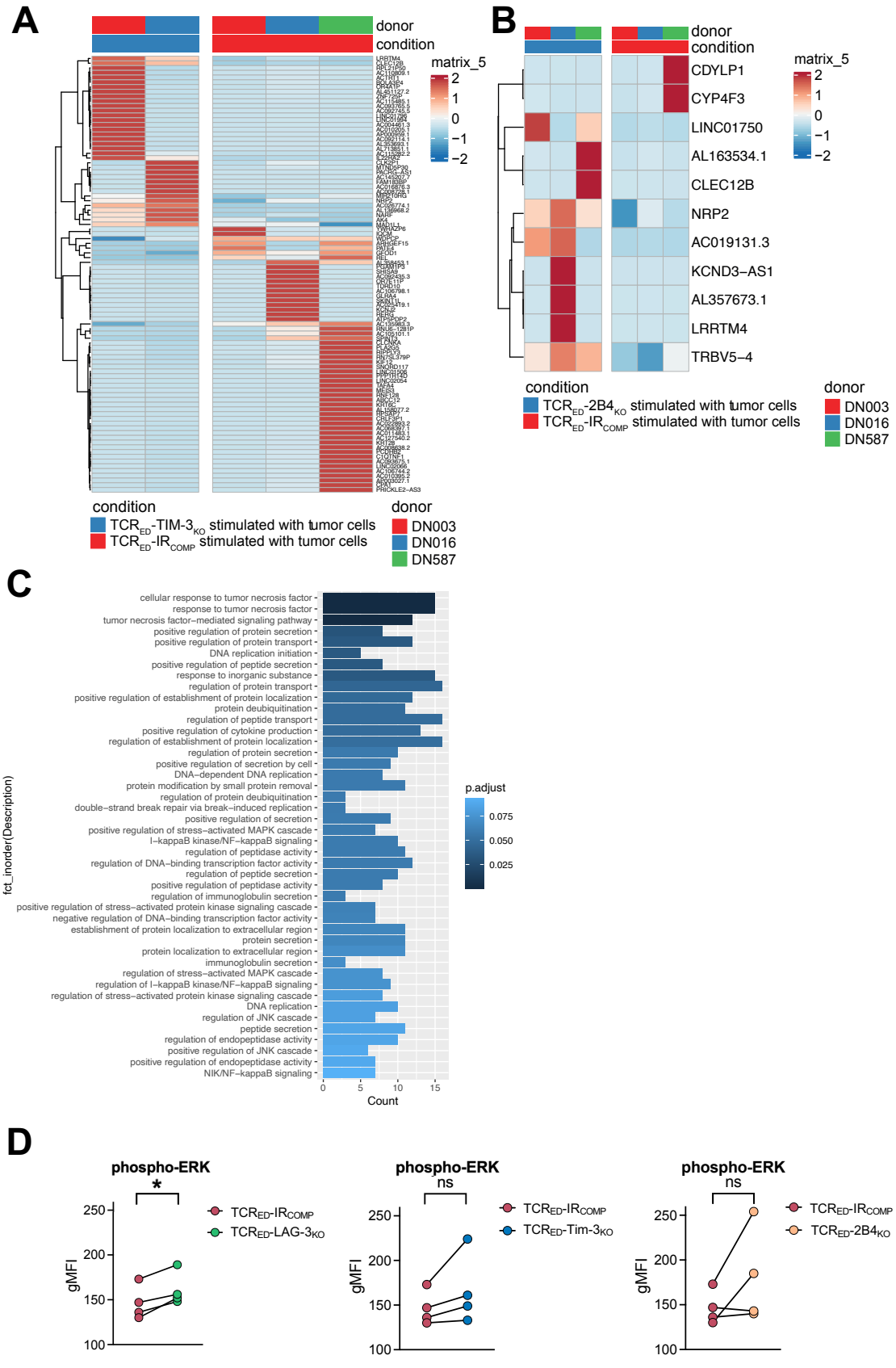
Supplementary Figure 4



Supplementary Figure 4. TCR_{ED}-IR_{KO} T cells produce higher amount of effector molecules than TCR_{ED}-IR_{COMP} T cells.

(**A**) Heatmap showing the ratio of secreted effector molecules produced by TCR_{ED}-IR_{KO} over TCR_{ED}-IR_{COMP} cells upon 24 hours co-culture with U266 (left panel) or HLA-$A2^{pos}ESO\text{-}1^{pos}$ MM1s target cells (right panel). Data are shown as mean ± SEM of at least 3 biological replicates. (**B**) Quantification of IFNγ produced by TCR_{ED}-IR_{COMP} (red bars), TCR_{ED}-LAG-3_{KO} (green bars), TCR_{ED}-TIM-3_{KO} (blue bars), and TCR_{ED}-$2B4_{KO}$ (light orange bars) T cells upon exposure to HLA-$A2^{pos}$ NY-ESO^{pos} U266 cells (left panel; N=6) or MM1.s $A2^{pos}$ $ESO\text{-}1^{pos}$ cells (right panel; N=3). Negative controls ($Ctrl^{neg}$) indicate exposure of effector cells to medium or wild-type MM1.s cells. Data are shown as mean ± SEM of 3-6 biological replicates.

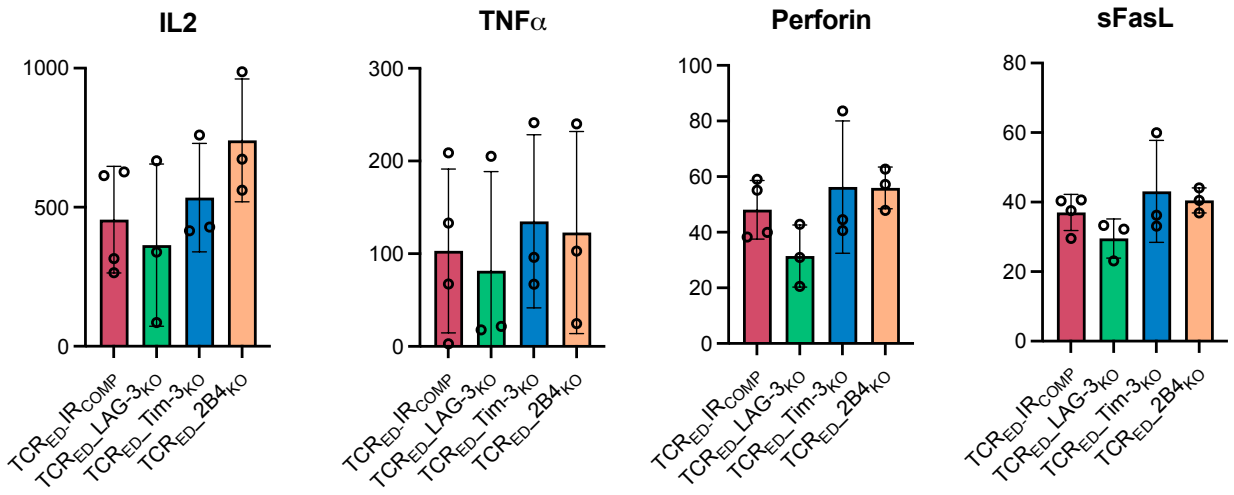
Supplementary Figure 5



Supplementary Figure 5. Gene expression analysis reveals specific transcriptomic changes in TCR_{ED}-IR_{KO} T cells.

Vulcano plots showing differentially expressed genes (DEGs) in TCR_{ED}-TIM-3_{KO} **(A)** and TCR_{ED}-$2B4_{KO}$ **(B)** T cells upon stimulation with MM1.s $A2^{pos}$ ESO-1^{pos} cancer cells. (**C**) Gene ontology (biological process) of the 30 uniquely DEGs in TCR_{ED}-$2B4_{KO}$ T cells upon stimulation with cancer cells. (**D**) Geometric mean fluorescence intensity (gMFI) of phospho-ERK measured by flow cytometry on TCR_{ED}-IR_{KO} and TCR_{ED}-IR_{COMP} cells 8 minutes after TCR triggering. Data obtained from 3 (panels A-C) and 4 (panel D) biological replicates are shown. *: p value <0.05; two-tailed paired t-test.

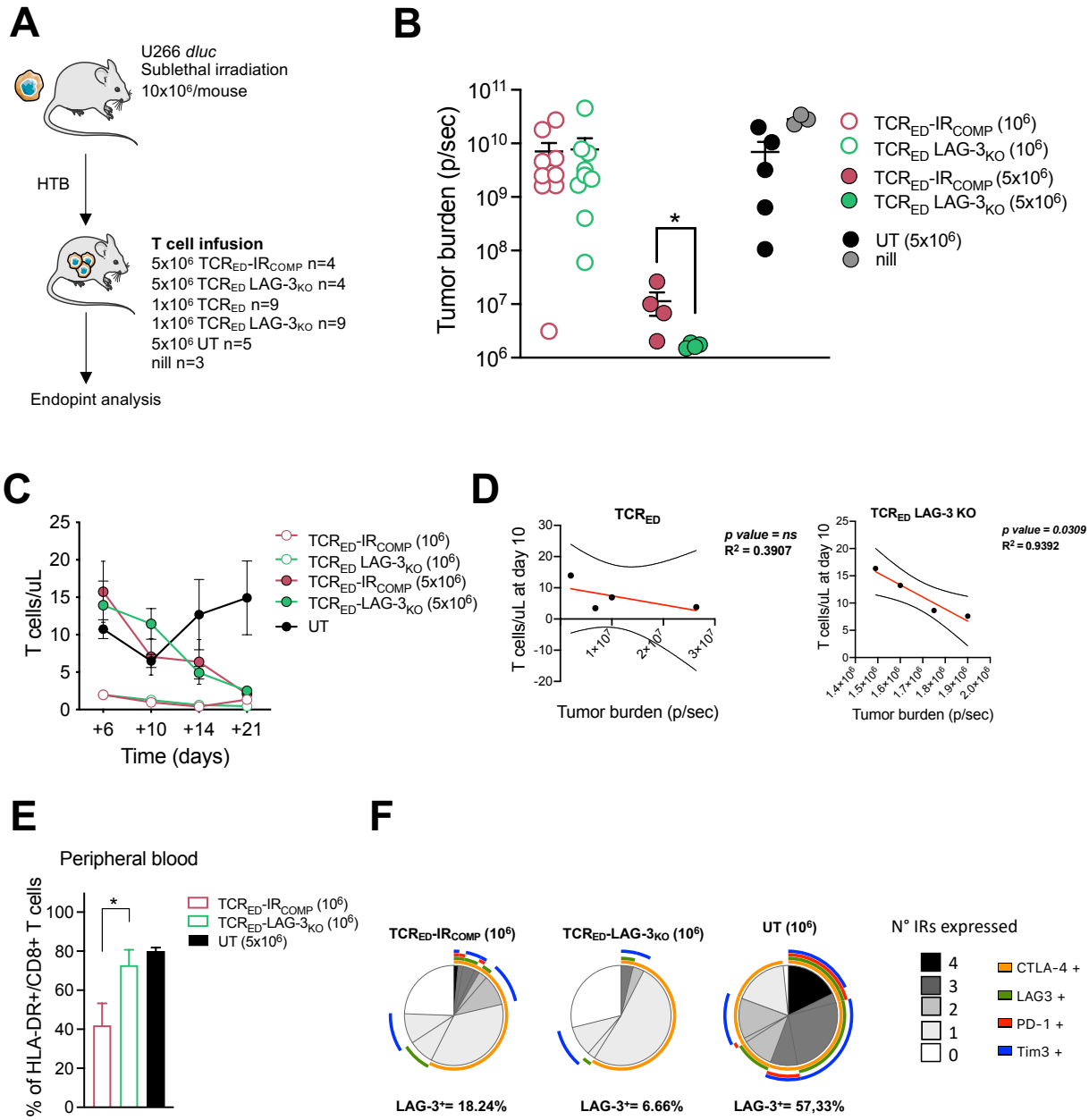
Supplementary Figure 6



Supplementary Figure 6. Similar cytokine secretion profile in TCR_{ED}-IR_{KO} and TCR_{ED}-IR_{COMP} T cells after chronic antigen stimulation

TCR_{ED}-IR_{COMP} and TCR_{ED}-IR_{KO} cells were daily stimulated with MM1.s $A2^{pos}ESO\text{-}1^{pos}$ cells as described in Figure 4A. Quantification of IL-2, TNFα, perforin, and sFasL produced by TCR_{ED}-IR_{COMP} (red bars; N=4), TCR_{ED}-$LAG\text{-}3_{KO}$ (green bars; N=3), TCR_{ED}-$TIM\text{-}3_{KO}$ (blue bars; N=3), and TCR_{ED}-$2B4_{KO}$ (light orange bars; N=3) upon exposure to HLA-$A2^{pos}$ NY-ESO^{pos} MM1.s $A2^{pos}ESO\text{-}1^{pos}$ cells is shown. Data were obtained from 3-4 biological replicates.

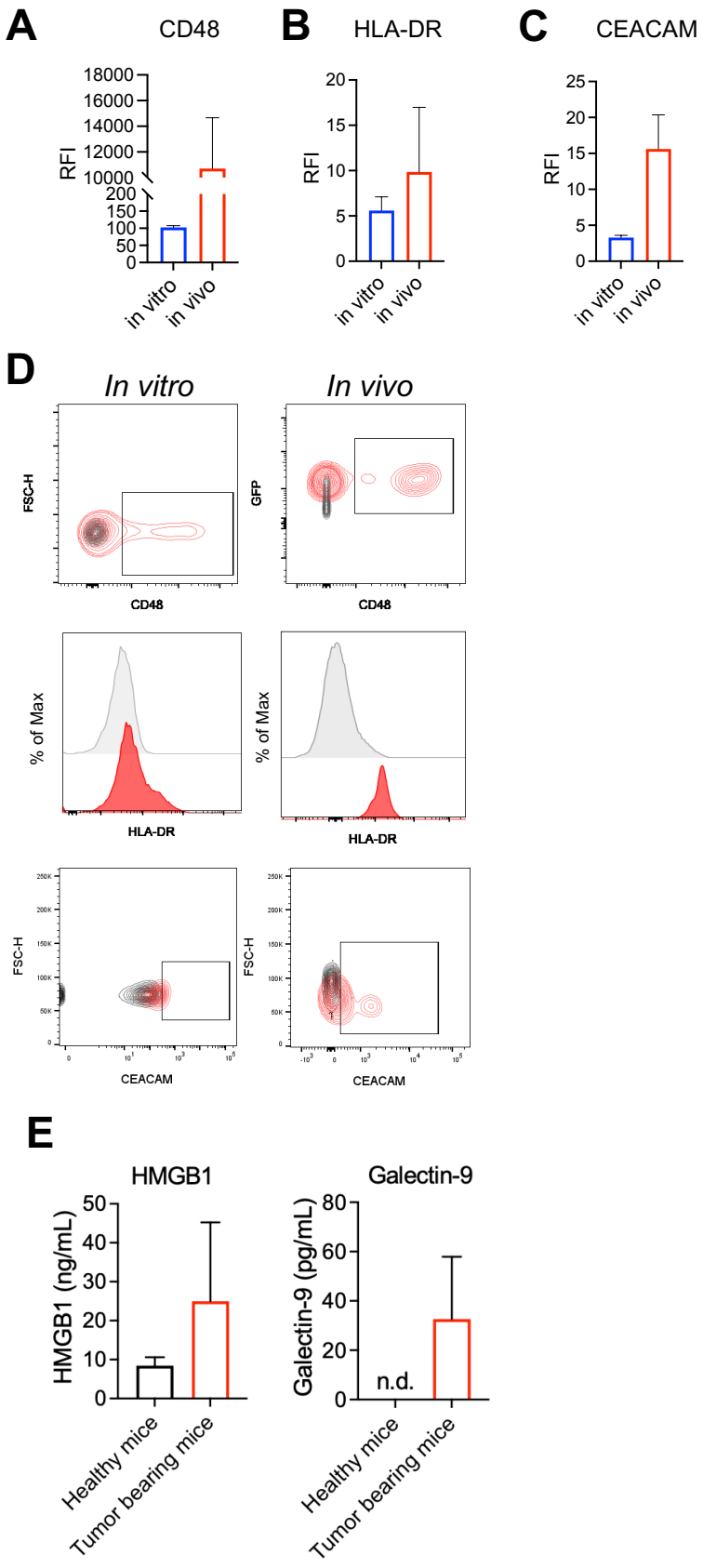
Supplementary Figure 7



Supplementary Figure 7. LAG-3_{KO} cells prevents the up-regulation of IRs in TCR_{ED} T cells *in vivo.*

(**A**) Schematic representation of *in vivo* high tumor burden (HTB) multiple myeloma model (U266). Tumor burden measured by total body bioluminescence, BLI (**B**) and absolute numbers of circulating human T cells (**C**) in mice treated with a low dose (empty circles) or a high dose (filled circles) of TCR_{ED}-IR_{COMP} (red) or TCR_{ED}-LAG-3_{KO} (green) cells or injected with a high dose of unmanipulated cells (UT, black) or left untreated (nil, gray). (**D**) Linear regression analysis of tumor burden and number of circulating T cells in treated mice. (**E**) Frequencies of activated (HLA-DR^{+}) T cells in the peripheral blood of mice treated with a low dose of TCR_{ED}-IR_{COMP} (red) or TCR_{ED}-LAG-3_{KO} (green) or UT cells (black). (**F**) Inhibitory receptors expression in T cells infiltrating the bone marrow of mice treated with low doses of TCR_{ED}-IR_{COMP} or TCR_{ED}-LAG-3_{KO} T cells or injected with UT cells. The slices in grayscale within the pie indicate the total number of co-expressed inhibitory receptors, while the external pie arches indicate the specific inhibitory receptor expressed. N. of treated and analyzed animals are shown in A.

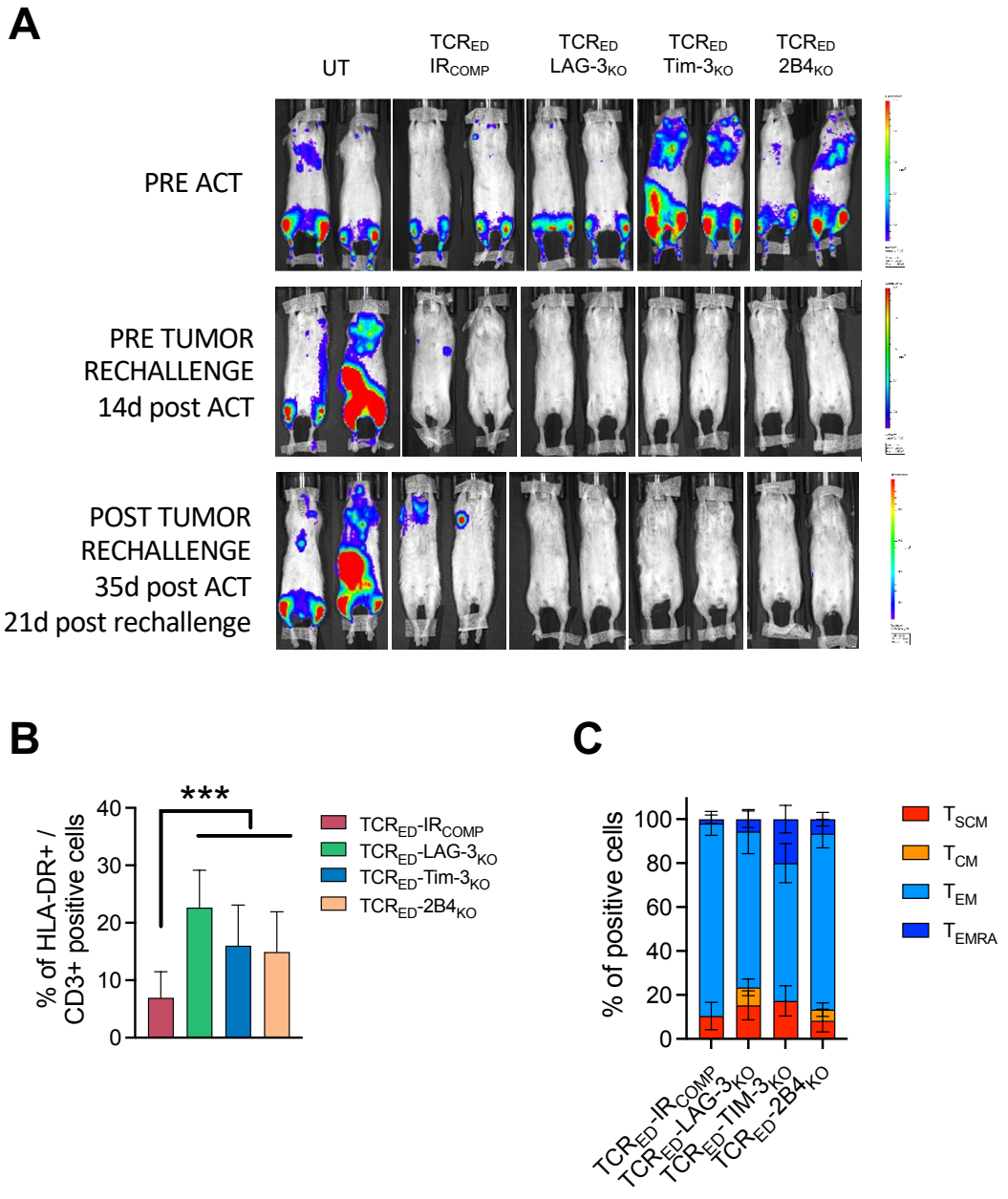
Supplementary Figure 8



Supplementary Figure 8. U266 cells upregulate IR ligands *in vivo*.

Quantification of CD48 (**A**), HLA-DR (**B**) and CEACAM (**C**) expressed *in vitro* or *in vivo* on the cell surface of U266 cells (N=3). RFI (ratio of fluorescence intensity) = Mean of fluorescence intensity of stained cells / Mean of fluorescence intensity of unstained cells. (**D**) Representative plots of CD48, HLA-DR and CEACAM expression on U266 cells. Unstained samples are shown in grey while stained samples are shown in red. (**E**) Quantification of galectin-9 and HMGB1 in the serum of U266 injected mice (N=3) and healthy mice (N=2), at sacrifice.

Supplementary Figure 9



Supplementary Figure 9. Activation and differentiation phenotypic profile of human T cells harvested in the rechallenge tumor model.

(A) Bioluminescence images of representative mice injected with U266 cells according to **Figure** 5A. Mice were analysed before adoptive cell therapy with TCR_{ED}-IR_{COMP} and TCR_{ED}-IR_{KO} cells (upper panels), after adoptive cell therapy, but before rechallenge with U266 cells (middle panels) and after rechallenge with the tumor. (**B**) Frequencies of activated (HLA-DR^{+}) $CD3^{+}$ T cells in the spleen at sacrifice after second tumor challenge. (**C**) Frequencies of stem memory T cells (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector cells (T_{EMRA}) in human CD3+ T cells in the spleen at sacrifice after second tumor challenge. Data are shown as mean $\pm$ SEM. N=6 for TCR_{ED}-IR_{COMP} (red), TCR_{ED}-LAG-3_{KO} (green), TCR_{ED}-TIM-3_{KO} (blue); N=5 TCR_{ED}-$2B4_{KO}$ (light orange).