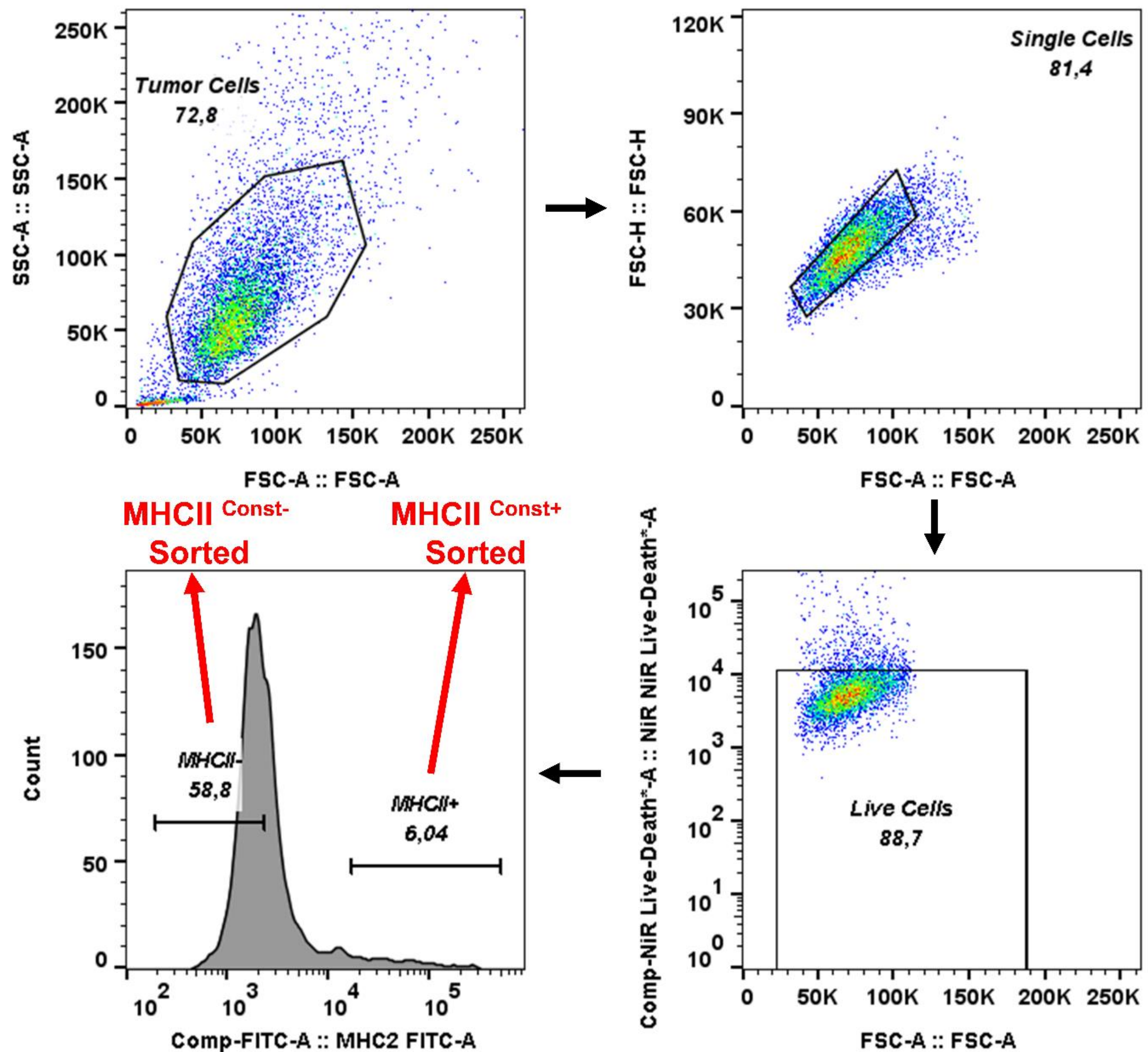
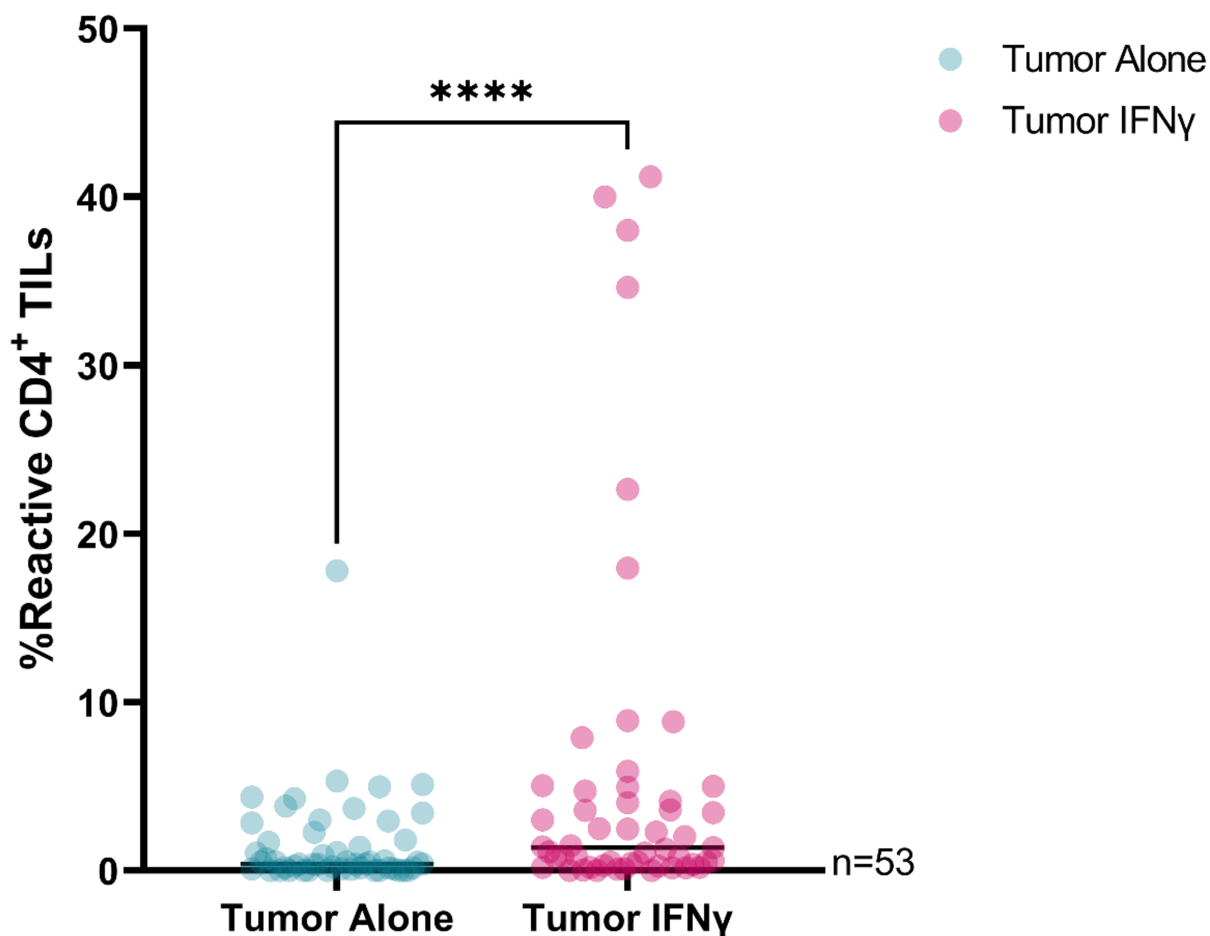
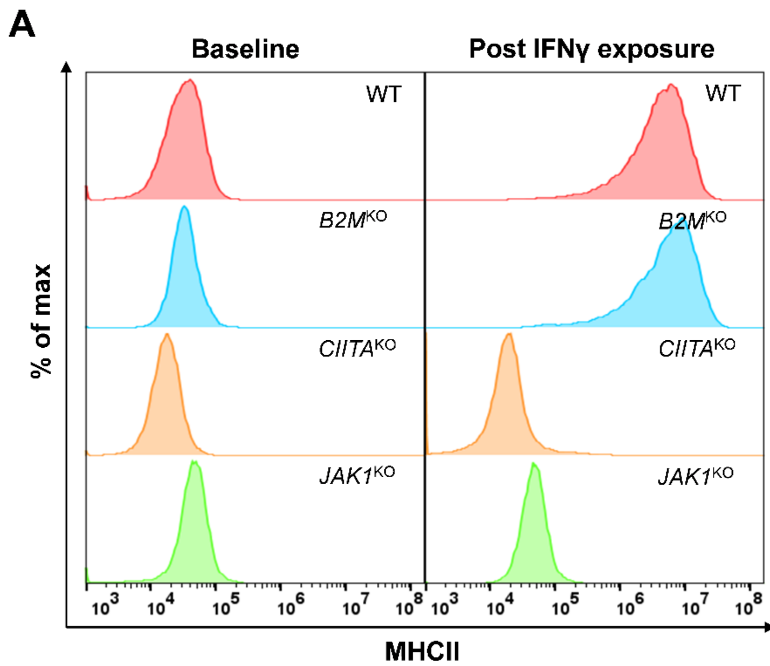
Supplementary Figures

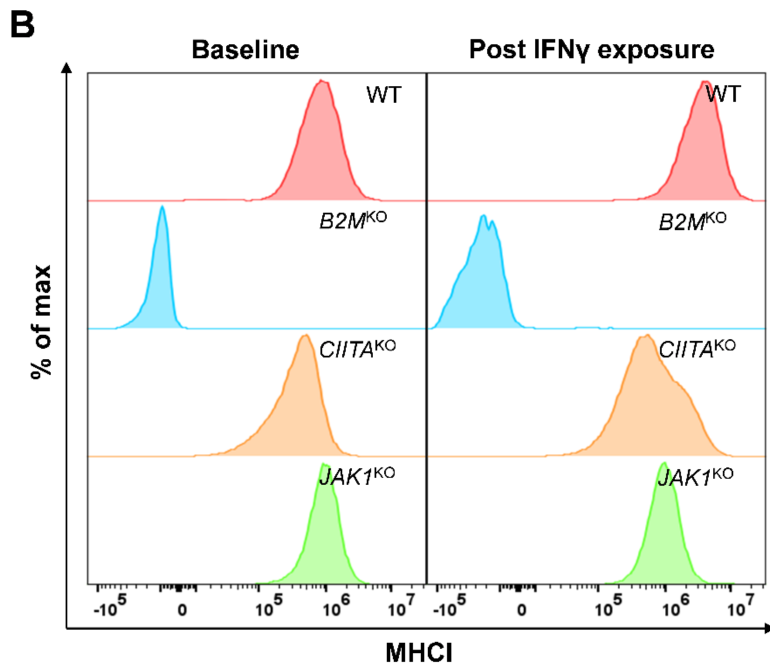


Supplementary Figure 1. MHCII^{const-} and MHCII^{const+} TCLs gating and sorting strategy. Tumor cells were selected based on a plot of FSC-A vs. SSC-A, and doublets were removed by gating FSC-A vs. FSC-H. Subsequently, cells negative for Live/Dead Fixable Dead Cell Stain Near-IR (NIR) were gated as live cells, and MHCII^{const-} and MHCII^{const+} tumor cells were gated in a plot of MHCII vs. Count. An isotype-stained control was used to set strict "MHCII^{const-}" and "MHCII^{const+}" gates.



Supplementary Figure 2. The antitumor reactivity of $CD4^+$ TILs against autologous TCLs is enhanced by tumor stimulation with IFNγ. The proportion of tumor-reactive $CD4^+$ TILs was significantly increased when the autologous TCLs were stimulated for 72 hours with IFNγ (Wilcoxon matched-pairs signed rank test, $p < 0.001$). The recognition of TILs was tested against separate sets of autologous tumor cells without previous IFNγ stimulation (tumor alone), and autologous tumor cells with previous 72-hour IFNγ stimulation (Tumor IFNγ). T cells were considered reactive if positive for at least one of TNF, IFNγ or CD107a, minus control. Horizontal lines illustrate median values. $p<0.0001$****





Supplementary Figure 3. *B2M*^{KO}, *CIITA*^{KO}, and *JAK1*^{KO} effect on MHCII and MHCI surface expression. (A-B) Representative plots showing **(A)** HLA–DR, DP, DQ and **(B)** HLA-A, B, C surface expression by flow cytometry in WT, *B2M*^{KO}, *CIITA*^{KO} and *JAK1*^{KO} TCL#17.

A

WT TCL#10 MHCII^{Const-}

B

WT TCL#10 MHCII^{Const-} IFNγ-stimulated

C

WT TCL#15 MHCII^{Const-}

D

WT TCL#15 MHCII^{Const-} IFNγ-stimulated

E

WT TCL#15 MHCII^{Const+}

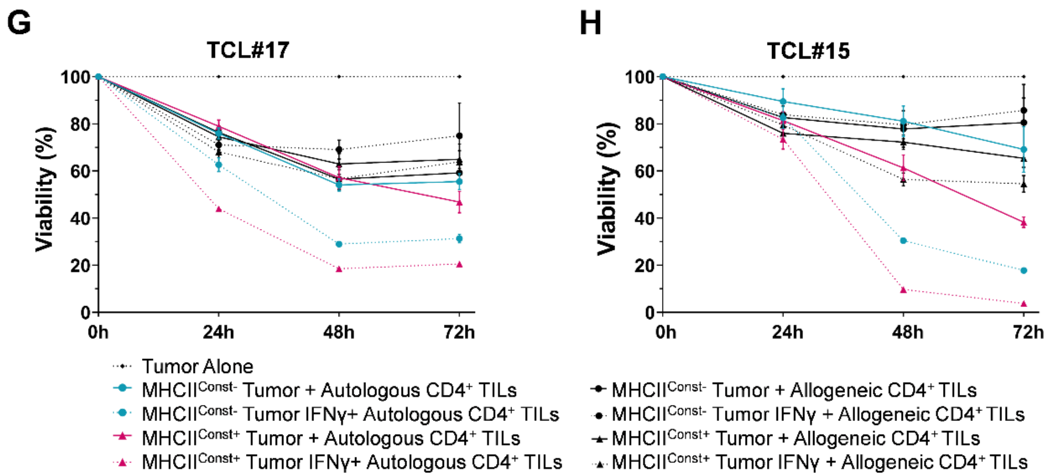
F

WT TCL#15 MHCII^{Const+} IFNγ-stimulated

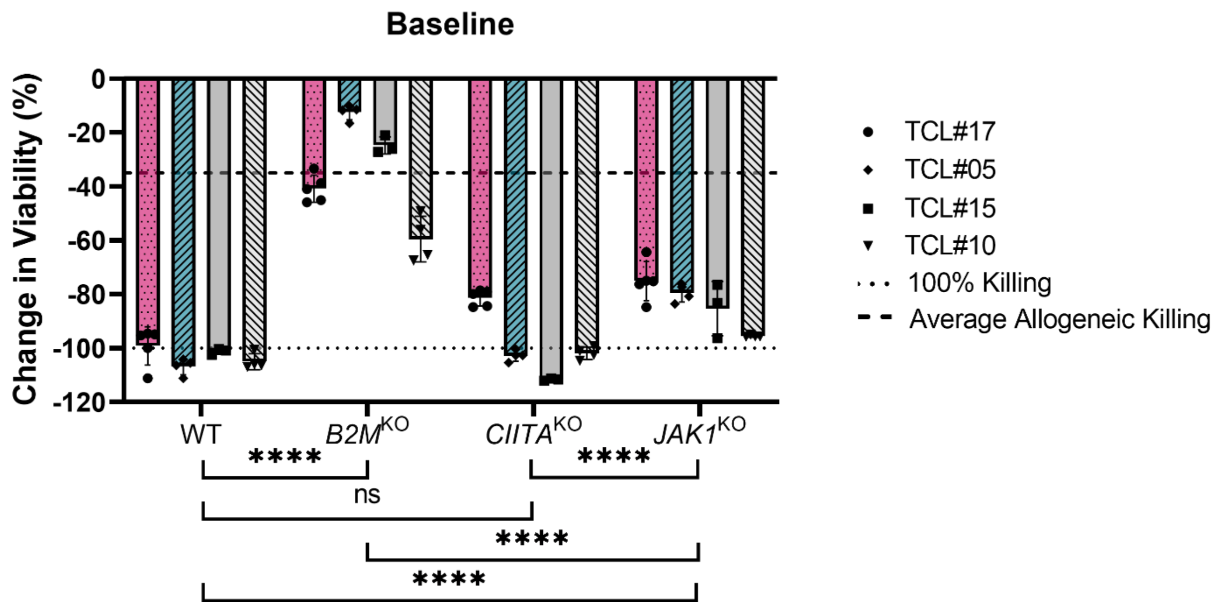
Normalized Cell Index

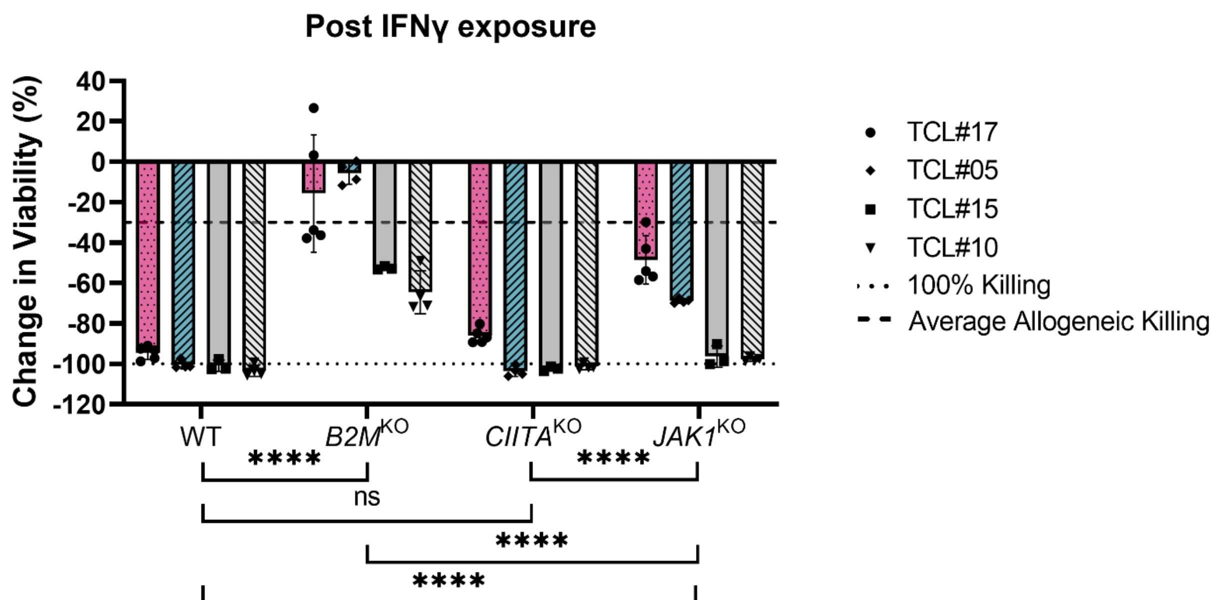
0h 24h 48h 72h

— Tumor Alone
— Tumor + Autologous CD4$^+$ TILs
— Tumor + Allogeneic CD4$^+$ TILs



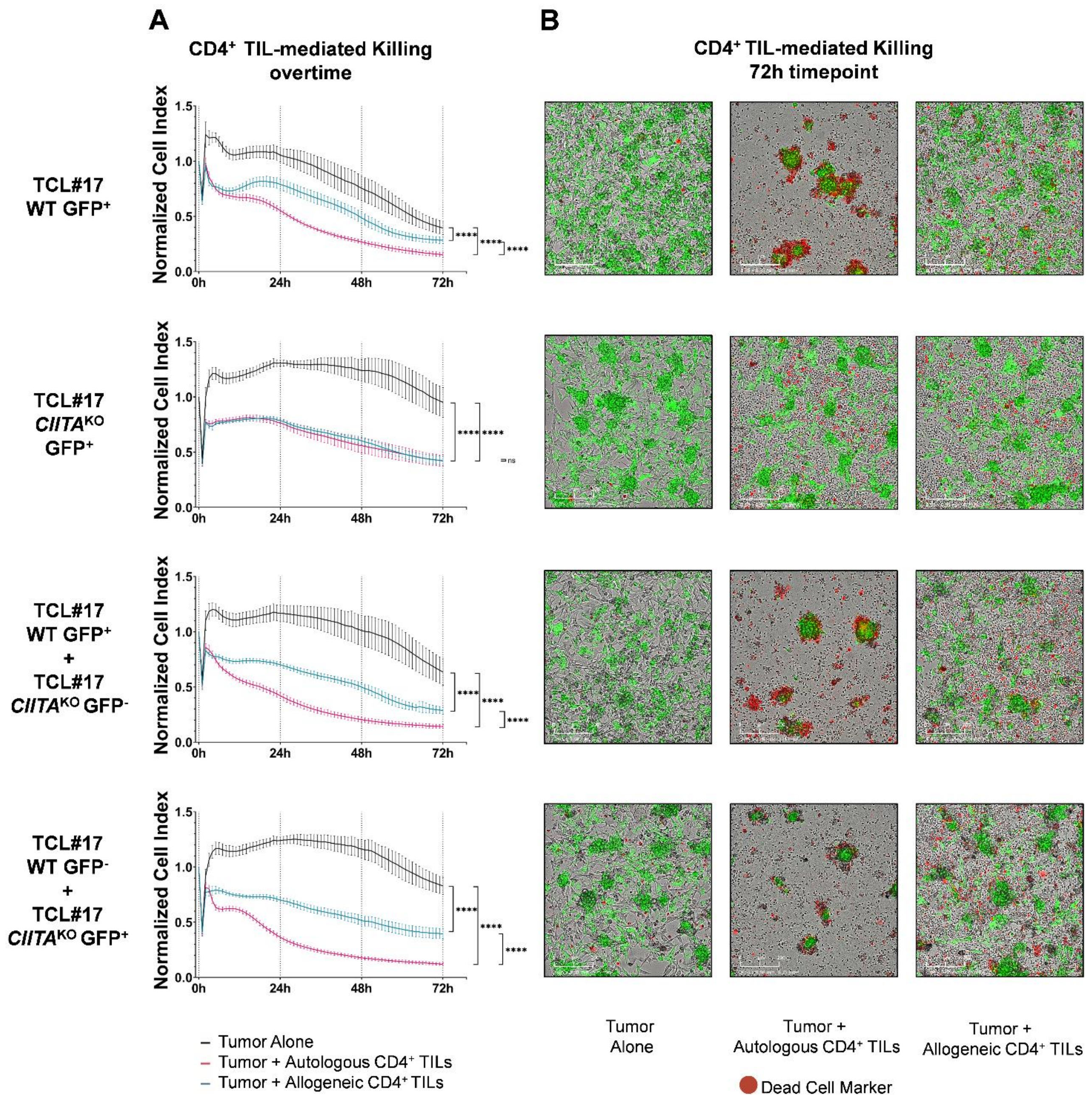
Supplementary Figure 4. Autologous CD4^{+} TIL-mediated cytotoxicity in TCL#10, TCL#15, and TCL#17. (A-F) Autologous CD4^{+}-TIL mediated killing at different time points during coculture with **(A-B)** TCL#10, **(C-D)** MHCII^{const-} TCL#15, and **(E-F)** MHCII^{const+} TCL#15 with and without previous 72-hour IFNγ stimulation. The black line represents the growth curve of the tumor alone, the red line represents the growth curve of the tumor in coculture with autologous CD4^{+} TILs, and the blue line represents the growth curve of the tumor in coculture with allogeneic CD4^{+} TILs (Effector:Target ratio 3:1). Autologous CD4^{+} TIL-mediated killing was considered specific if a significant difference between both autologous CD4^{+} TIL and tumor alone curves and autologous CD4^{+} TIL and allogeneic CD4^{+} TIL curves was observed. Plot A represents the same control condition shown for TCL#10 in Figure 3B. Tukey's multiple comparisons test was employed to compare the different curves. Data are presented as mean ± SD. $p<0.001$***; $p<0.0001$****; ns=not significant; **(G-H)** Autologous CD4^{+} TIL-mediated killing at different time points during co-culture with MHCII^{const+} and MHCII^{const-} **(G)** TCL#17 and **(H)** TCL#15 with and without previous 72-hour IFNγ stimulation. Each line represents the relative change in tumor viability (%) compared to the matched tumor alone condition during coculture with autologous CD4^{+} TILs (Effector:Target ratio 3:1). Data are presented as mean ± SD. Data are representative of two to three independent experiments.





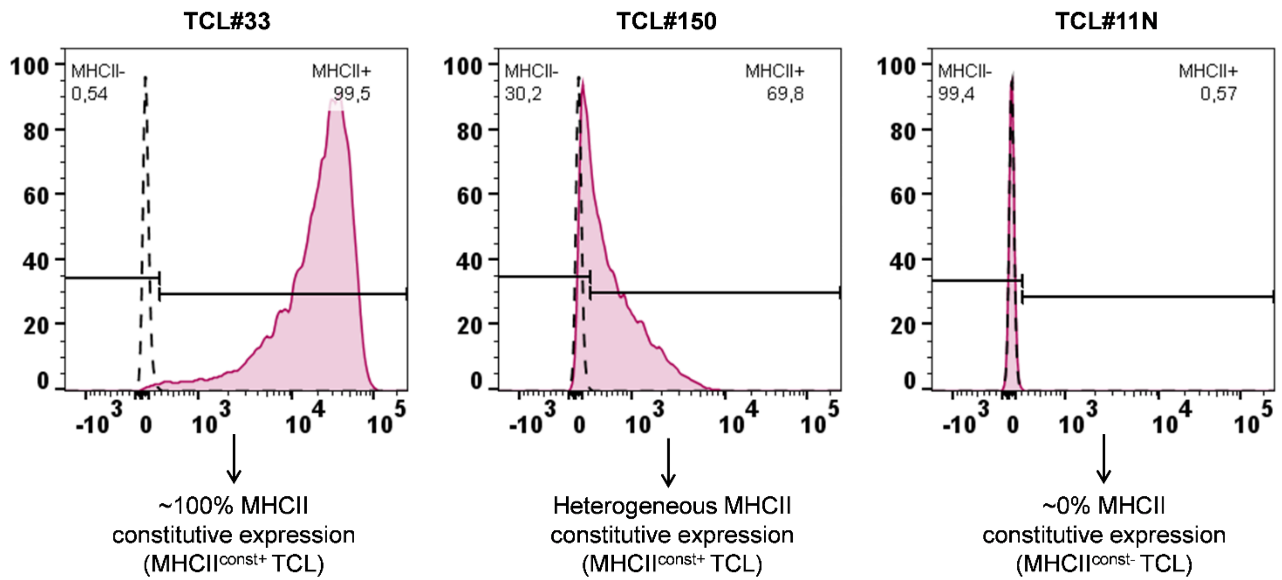
Supplementary Figure 5. ***B2M*^KO^, C*IITA*^KO^, and *JAK1*^KO^ effect on CD8^+^ TIL-mediated killing.** **(A-B)** Autologous CD8^+^ TIL-mediated killing at 72h during coculture with WT, *B2M*^KO^, C*IITA*^KO^, and *JAK1*^KO^ TCLs **(A)** without and **(B)** with previous 72-hour IFNγ stimulation. Each bar represents the relative change in tumor viability (%) compared to the matched tumor alone condition during coculture with autologous CD8^+^ TILs (Effector:Target ratio 3:1). *B2M*^KO^ and *JAK1*^KO^ significantly affected the autologous CD8^+^ TIL-mediated killing, however, *JAK1*^KO^ did not completely abrogate it

(Wilcoxon matched-pairs signed rank test, $p<0.001$***, ns=not significant). Each dot represents a technical replicate. Data are presented as mean ± SD.

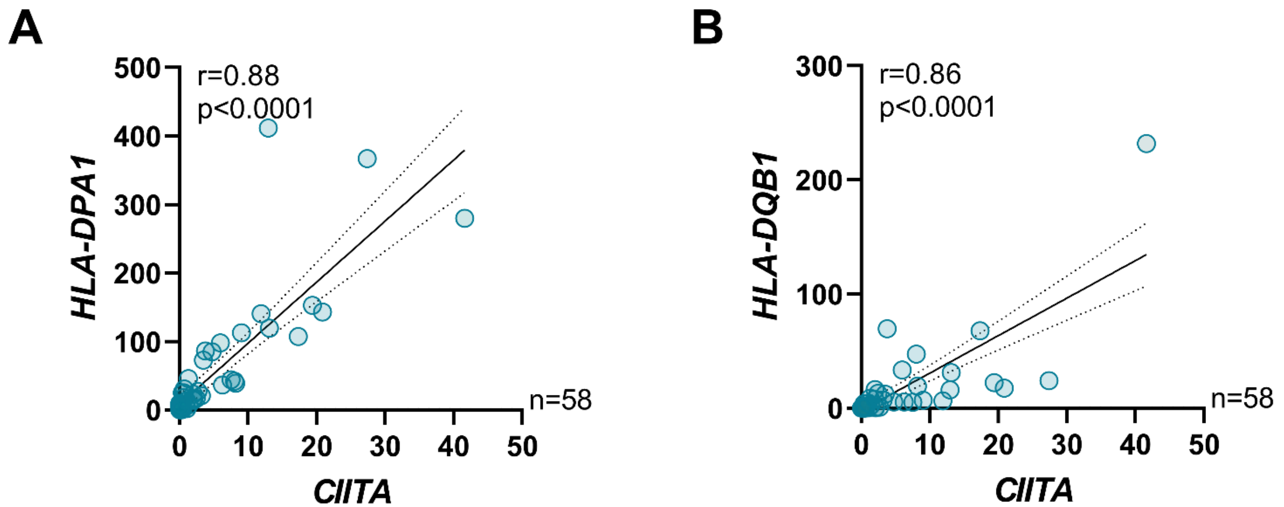


Supplementary Figure 6. CD4$^+$ TIL-mediated cytotoxicity against MHCII$^+$ tumor cells can trigger cytotoxicity against MHCII$^-$ tumor cells. **(A-B)** Each row represents a different TCL or a combination of MHCII$^+$ and MHCII$^-$ TCLs derived from TCL#17 **(A)** CD4$^+$-TIL mediated killing at different time points during coculture with WT GFP$^+$ TCL#17, or *CIITA*^{KO} GFP$^+$TCL#17, or WT GFP$^+$ TCL#17 plus *CIITA*^{KO} GFP- TCL#17 (MHCII$^+$ TCL:MHCII$^-$ TCL ratio 1:1), or WT GFP$^-$ TCL#17 plus *CIITA*^{KO} GFP$^+$ TCL#17 (MHCII$^+$ TCL:MHCII$^-$ TCL ratio 1:1), with previous IFNγ

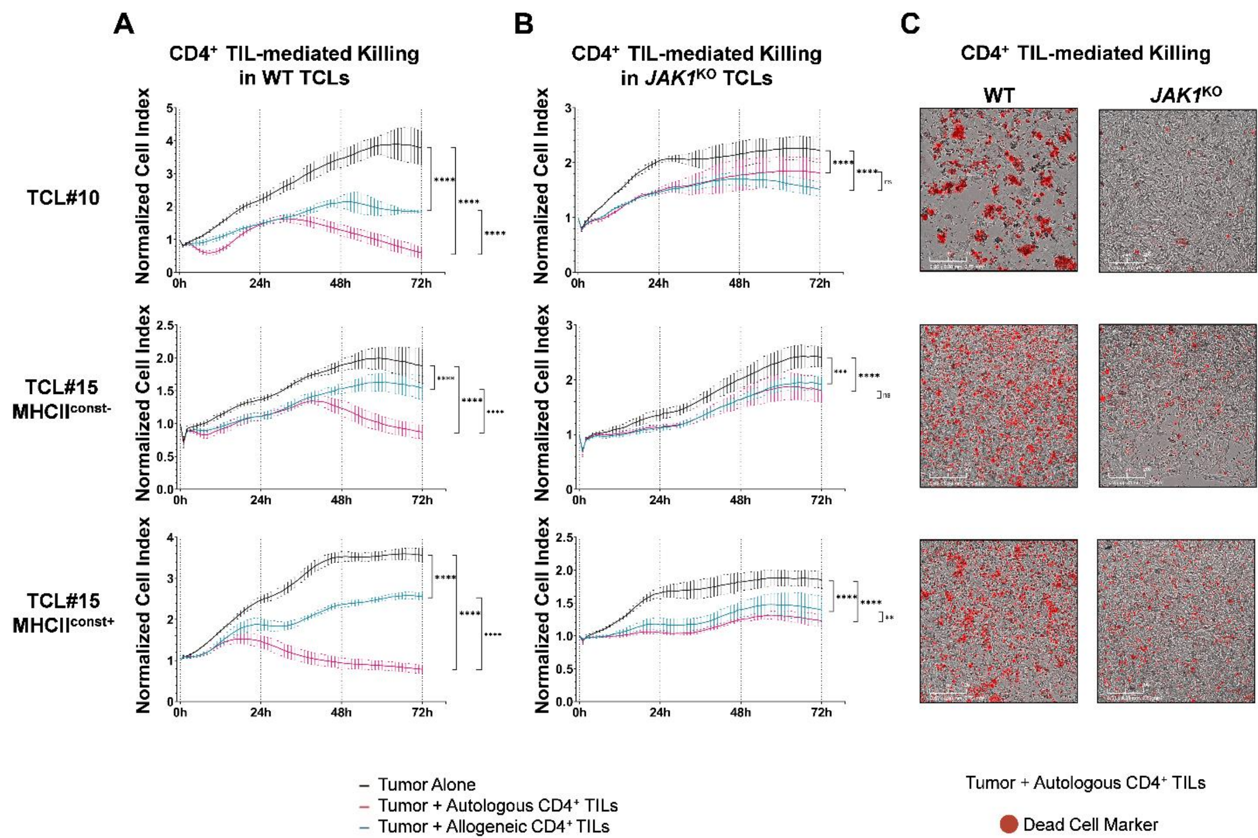
stimulation. Growth curves are colored in black (tumor alone), red (tumor in coculture with autologous $CD4^+$ TILs), or blue (tumor in coculture with allogeneic $CD4^+$ TILs) (Effector:Target ratio 3:1). Autologous $CD4^+$ TIL-mediated killing was considered specific if a significant difference between both autologous $CD4^+$ TIL and tumor alone curves and autologous $CD4^+$ TIL and allogeneic $CD4^+$ TIL curves was observed. Tukey's multiple comparisons test was employed to compare the different curves. Data are presented as mean ± SD. p<0.0001****; ns=not significant. Except for the *CIITA*^{KO} tumor, no detectable killing differences were observed across the other conditions. **(B)** Representative images of the tumors alone (left panels), tumors in coculture with autologous $CD4^+$ TILs (middle panels), and tumors in coculture with allogeneic $CD4^+$ TILs (right panels) after 72 hours of coculture (Effector:Target ratio 3:1). The eTox red reagent was used to stain the nuclei of dead cells and to detect cytotoxicity. Overall, after 72 hours of coculture, the *CIITA*^{KO} tumor remained alive, while neither the WT tumor nor any of the two subpopulations in the mixed TCL conditions (WT or *CIITA*^{KO}) survived.



Supplementary Figure 7. Phenotypes of constitutive MHCII expression. HLA–DR, DP, and DQ constitutive surface expression by flow cytometry in three representative TCLs (TCL#33, TCL#150, and TCL#11N) characterized by different MHCII expression phenotypes. The black dotted line represents HLA–DR, DP, and DQ expression when tumor cells were stained with isotype control. The red line represents constitutive surface expression of HLA–DR, DP, and DQ (i.e., without previous 72-hour IFNγ stimulation).

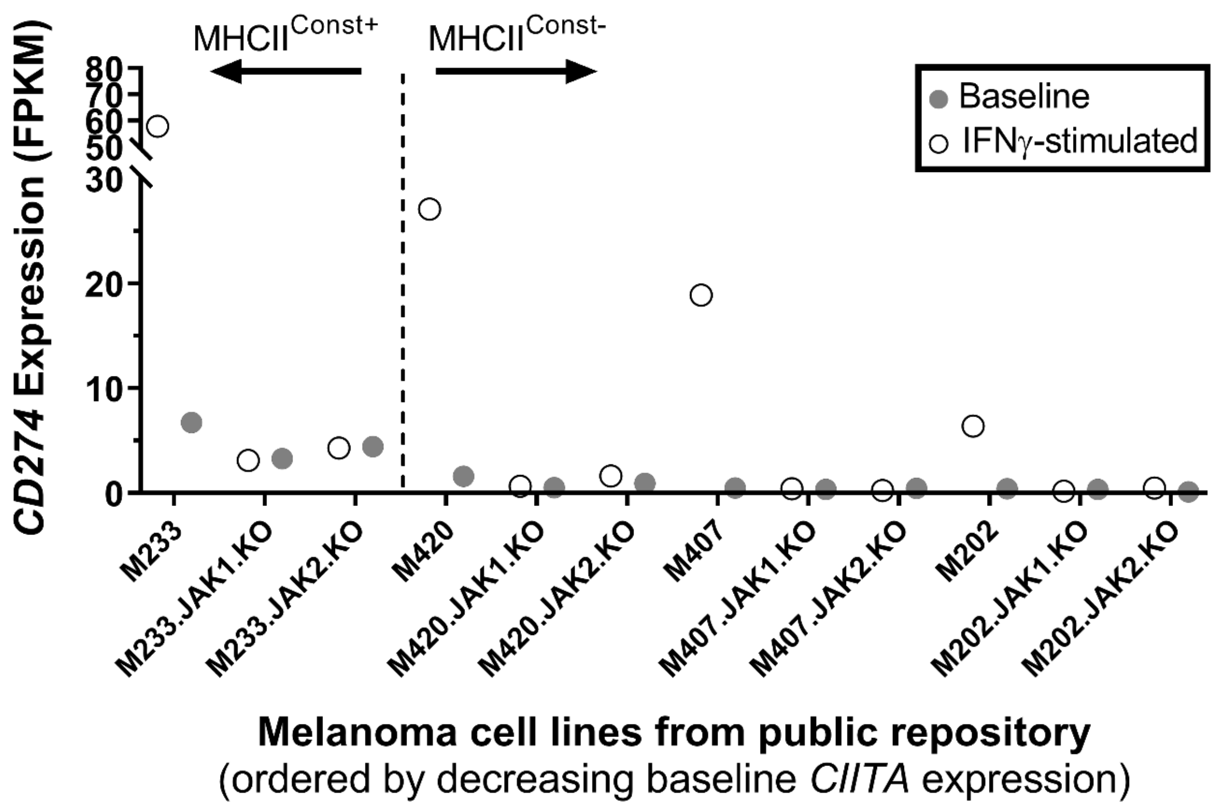


Supplementary Figure 8. *CIITA* expression strongly correlates to MHCII genes expression. (A-B) Correlation between *CIITA* and **(A)** *HLA-DPA1* or **(B)** *HLA-DQB1* expression in 58 human melanoma TCLs without IFNγ stimulation. *CIITA* expression was strongly correlated to *HLA-DPA1* (Spearman r = 0.88, p < 0.0001) and *HLA-DQB1* (Spearman r = 0.86, p < 0.0001) expression. The solid line and dotted lines represent the best-fit regression line and 95% confidence interval, respectively. A bulk RNAseq dataset (1) from public repositories was re-analyzed to produce this figure.



Supplementary Figure 9. *JAK1^KO* abrogates CD4$^+$ TIL-mediated cytotoxicity against MHCII^{const-}, but not against MHCII^{const+} melanoma TCLs. (A-B) Autologous CD4$^+$-TIL mediated killing at different time points during coculture with **(A)** WT and **(B)** *JAK1*^{KO} TCLs with previous 72-hour IFNγ stimulation. The black line represents the growth curve of the tumor alone, the red line represents the growth curve of the tumor in coculture with autologous CD4$^+$ TILs, and the blue line represents the growth curve of the tumor in coculture with allogeneic CD4$^+$ TILs (Effector:Target ratio 3:1). Autologous CD4$^+$ TIL-mediated killing was considered specific if a significant difference between both autologous CD4$^+$ TIL and tumor alone curves and autologous CD4$^+$ TIL and allogeneic CD4$^+$ TIL curves was observed. Figure B (TCL#10) represents the same experimental condition shown in Supplementary Figure 4B, here used as a control condition. Tukey's multiple comparisons test was employed to compare the different curves. Data are presented as mean ± SD. $p<0.001$***;

p<0.0001****; ns=not significant. **(C)** Representative pictures of the autologous CD4^{+}-TIL mediated killing at 72 hours during coculture with WT and *JAK1*^{KO} TCLs (Effector:Target ratio 3:1). The eTox red reagent was used for detection of cytotoxicity. Data are representative of two to three independent experiments. The HLA–DR, DP, DQ surface expression by flow cytometry in WT and *JAK1*^{KO} TCL#10, TCL#15 MHCII^{const+}, and TCL#15 MHCII^{const-} with and without previous 72-hour IFNγ stimulation is shown in Figure 3A.



Supplementary Figure 10. Constitutive expression of PD-L1 in MHCII^{const+} TCLs with alterations in the IFNγ-signaling pathway could indicate a potential role for ICI treatment in tumors with *JAK1/2* mutations. Effect of IFNγ on *CD274* gene expression in 4 human melanoma TCLs. TCLs were arranged from left to right according to decreasing baseline *CIITA* expression (without IFNγ stimulation). For each melanoma, WT, *JAK1*^{KO} and *JAK2*^{KO} TCLs are shown. Full dots represent *CD274* expression without IFNγ stimulation, while empty dots represent *CD274* expression post 6-hour IFNγ stimulation. A bulk RNAseq dataset (1) from public repositories was re-analyzed to produce this figure.

Supplementary Tables

Supplementary Table 1: Flow-cytometry antibody panels.

Panel purpose	Staining phase	Target	Fluorochrome	Clone	Provider	Cat. No.	Purpose
MHC class I and class II staining on tumor cells	Surface staining	HLA–DR, DP, DQ	FITC	Tu39	BD Biosciences	555558	MHCII Stain
		HLA-ABC	APC	G46-2.6	BD Biosciences	555555	MHCI Stain
		Mouse IgG2a, κ	FITC	Not Applicable	BD Biosciences	555573	Isotype Control
		Mouse IgG1a, κ	APC	Not Applicable	BD Biosciences	555751	Isotype Control
		Dead cells	APC-Cy7	Not Applicable	Thermo Fisher Scientific	L34976	Live/dead
TIL reactivity assays	Coculture	CD107a	BV421	H4A3	BD Biosciences	562623	Degranulation
	Surface staining	CD3	PE-CF594	UCHT1	BD Biosciences	562280	T cell Lineage
		CD56	BV510	NCAM16.2	BD Biosciences	563041	NK cell Lineage
		CD4	BV711	SK3	BD Biosciences	563028	T cell Lineage
		CD8	Qdot605	3B5	Thermo Fisher Scientific	Q10009	T cell Lineage
		Dead cells	APC-Cy7	Not Applicable	Thermo Fisher Scientific	L34976	Live/dead
	Intracellular staining	TNF	APC	MAb11	BD Biosciences	554514	Activation
		IFNγ	PE-Cy7	B27	BD Biosciences	557643	Activation
		CD137	PE	4B4-1	BD Biosciences	555956	Activation

Supplementary Table 2: Antitumor reactivity of $CD4^{+}$ TILs against autologous TCLs prestimulated or not with IFNγ.

Patient ID	Tumor Alone	Tumor IFNγ
TCL#02	0,40	0,11
TCL#04	0,02	0,16
TCL#05	2,83	3,60
TCL#06	1,05	3,44
TCL#071	0,00	0,85
TCL#09	0,00	0,09
TCL#10	0,23	17,97
TCL#11O	0,90	1,06
TCL#12	17,80	41,20
TCL#131	0,15	4,95
TCL#14	0,38	0,43
TCL#15	0,00	34,64
TCL#150	1,08	4,12
TCL#17	0,02	40,01
TCL#172	0,50	0,20
TCL#24	1,68	3,01
TCL#26	5,31	5,04
TCL#27	1,81	38,01
TCL#301	0,27	1,36
TCL#303	0,10	2,03
TCL#305	0,16	3,59
TCL#306	0,57	0,77
TCL#307	0,00	0,16
TCL#311	3,01	2,50
TCL#312.1	0,18	0,19
TCL#312.2	0,07	0,59
TCL#313	0,54	1,11
TCL#314	0,01	7,90
TCL#315	2,95	2,47
TCL#317	0,00	1,47
TCL#322	0,10	0,03
TCL#33	5,12	4,74
TCL#34	0,00	0,00
TCL#36	3,42	1,28
TCL#401	0,11	0,19
TCL#405	0,37	0,84
TCL#41	0,40	0,37
TCL#411	0,37	0,27
TCL#412	0,40	0,30

TCL#413	4,29	4,02
TCL#43.2	4,37	8,84
TCL#47	0,71	1,41
TCL#601	4,97	5,90
TCL#605	1,40	5,00
TCL#611	0,50	0,50
TCL#833	0,17	0,00
TCL#904	0,00	0,00
TCL#914	3,70	8,90
TCL#915	3,82	22,63
TCL#M02	0,00	0,77
TCL#M04	0,50	0,50
TCL#M05	0,50	0,50
TCL#M10	2,27	2,29
Mean	1,50	5,52
Median	0,40	1,36

T cells were considered reactive if positive for at least one of TNF, IFNγ, or CD107a, minus control. TCLs were considered able to induce $CD4^+$ TIL reactivity if more than 0.5% of the autologous $CD4^+$ TILs were reactive.

References

1. Grasso CS, Tsoi J, Onyshchenko M, Abril-Rodriguez G, Ross-Macdonald P, Wind-Rotolo M, *et al.* Conserved Interferon-gamma Signaling Drives Clinical Response to Immune Checkpoint Blockade Therapy in Melanoma. Cancer Cell **2020**;38:500-15 e3