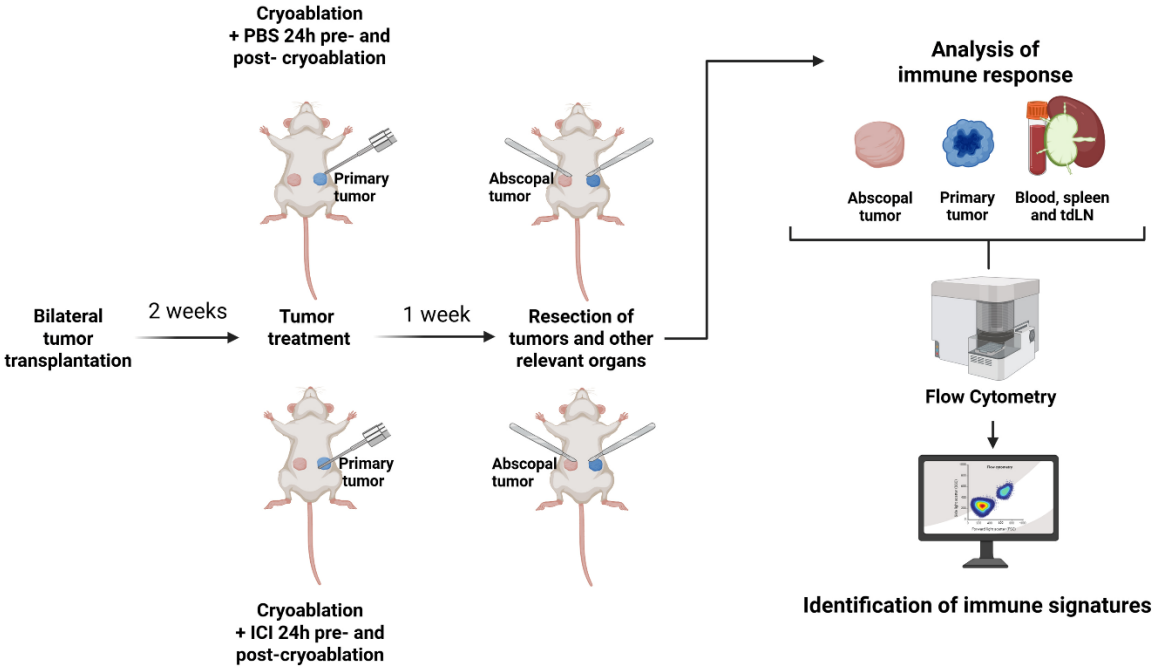
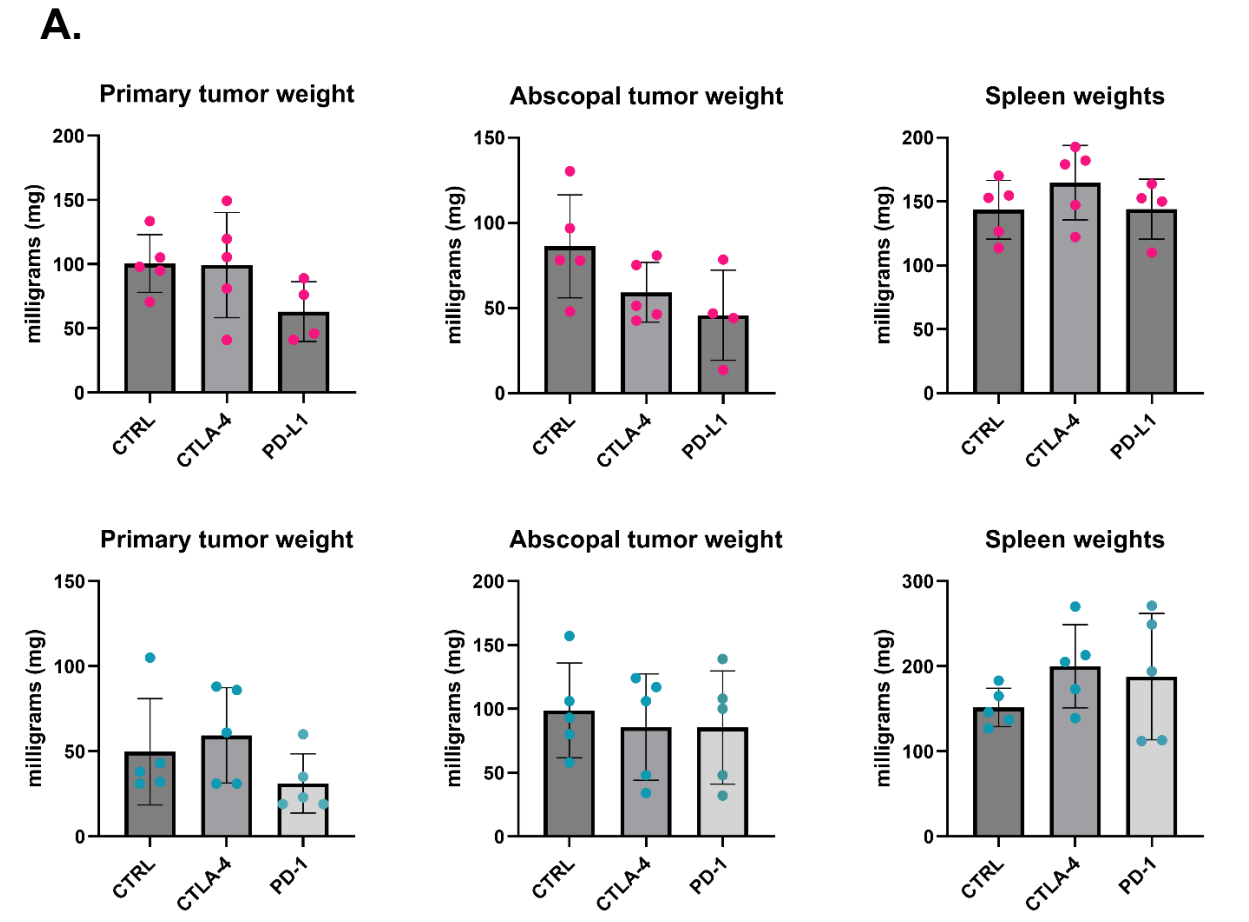
Supplementary Materials

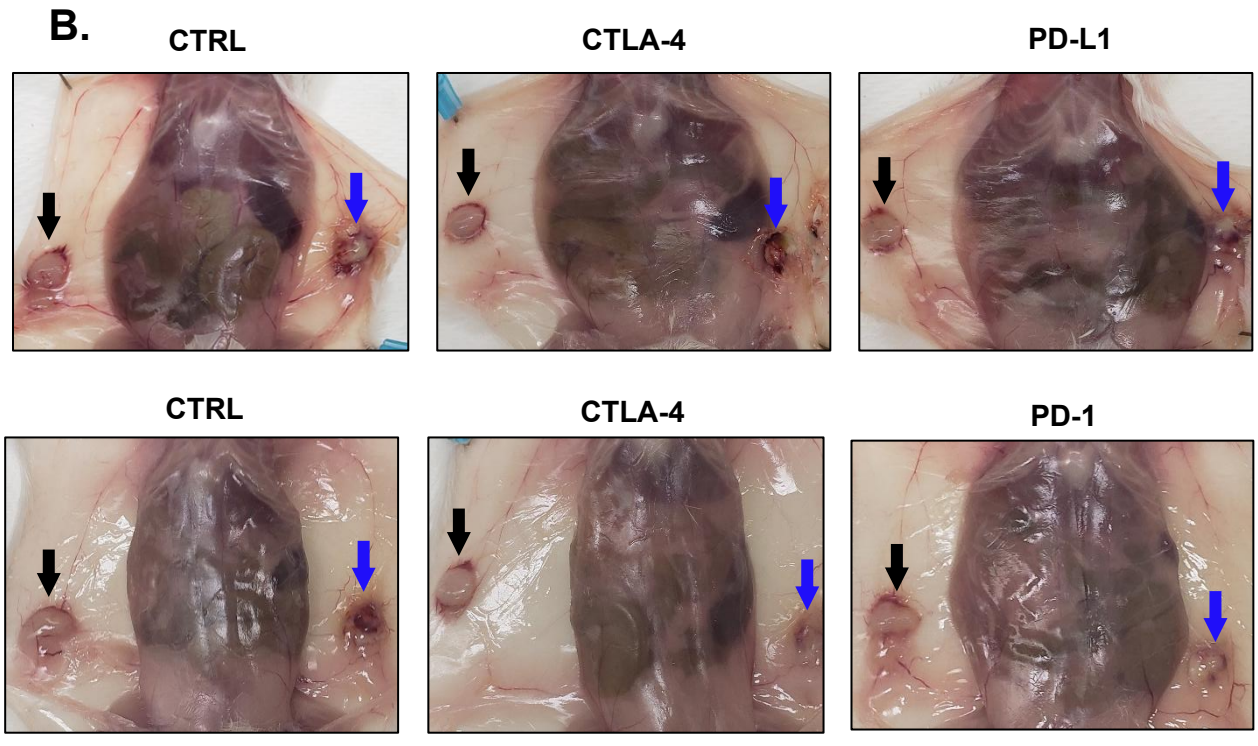
Supplementary figure 1.



Supplementary figure 1. Experimental approach. Naïve BALB/c female mice received a bilateral orthotopic transplantation of one million 4T1-12B luciferase expressing cells and, two weeks later, primary (left) tumors were cryoablated; abscopal (right) tumors were unmanipulated. Twenty-four hours pre- and post-cryoablation, mice were administered PBS or ICIs via intraperitoneal injections. A week after, mice were sacrificed and peripheral blood, spleen, tumors and tumor-draining lymph nodes were collected. Organs were processed to obtain single cells for flow cytometry analysis. Created in BioRender. Sardela de Miranda, F. (2025) https://BioRender.com/676r9hv

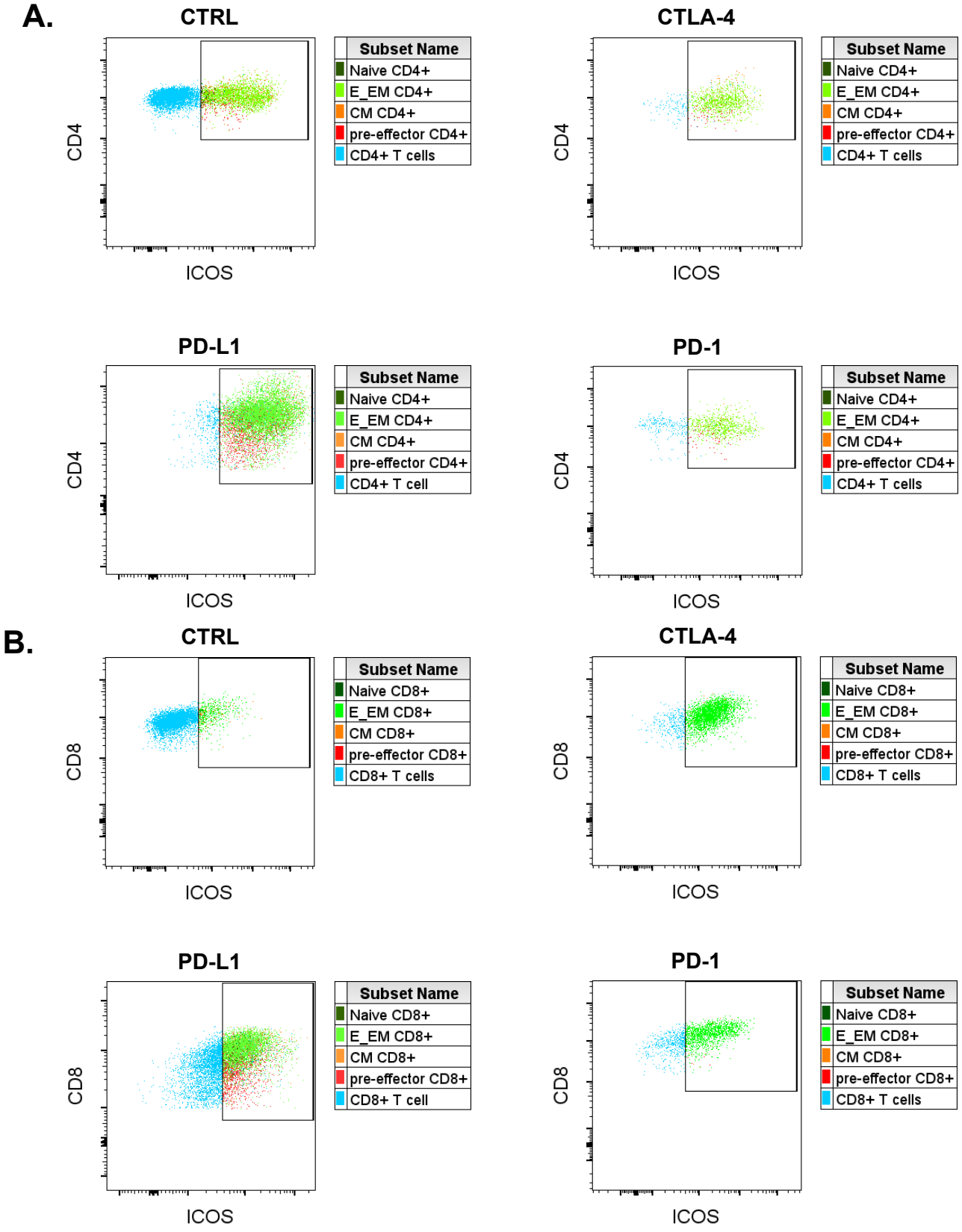
Supplementary figure 2.





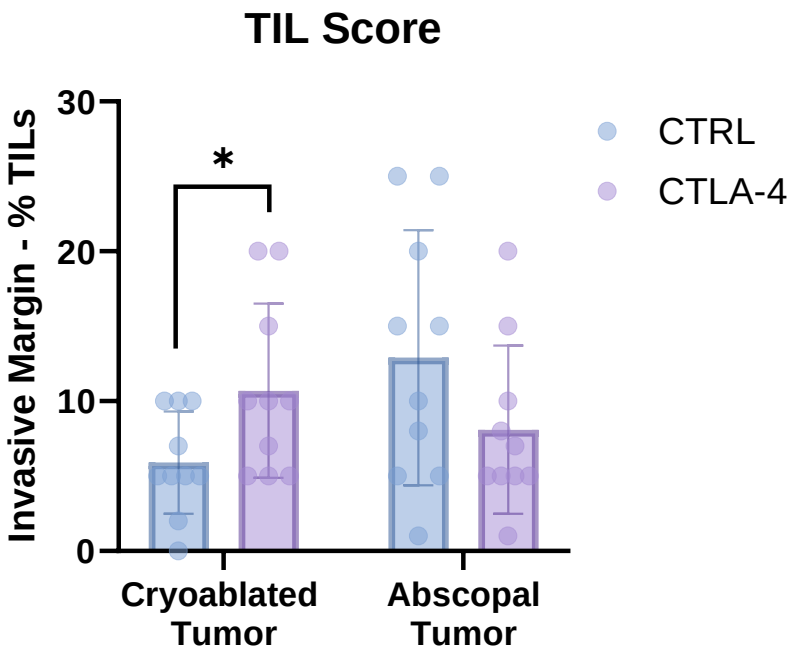
Supplementary figure 2. Organ weights and tumor size between the different groups one week after cryoablation. (A) After euthanasia, mouse tumors and spleen were collected and weighed. Pink and blue dots (Top and bottom, respectively), indicate different replicates of the experiment. (B) Representative pictures of Cryoablated tumors (blue arrow) and abscopal tumors (black arrow) in the multiple groups in different replicates of the experiment (Top and bottom, respectively). One-way ANOVA for normally distributed data or Kruskal-Wallis test for non-normally distributed data was performed comparing cryoablation monotherapy to combination with ICIs, with $p<0.05$ considered significant. Trends are shown where relevant. CTRL = control, n = 10; CTLA-4 = anti-CTLA-4, n = 10; PD-L1 = anti-PD-L1, n = 5; PD-1 = anti-PD-1, n = 5.

Supplementary figure 3.



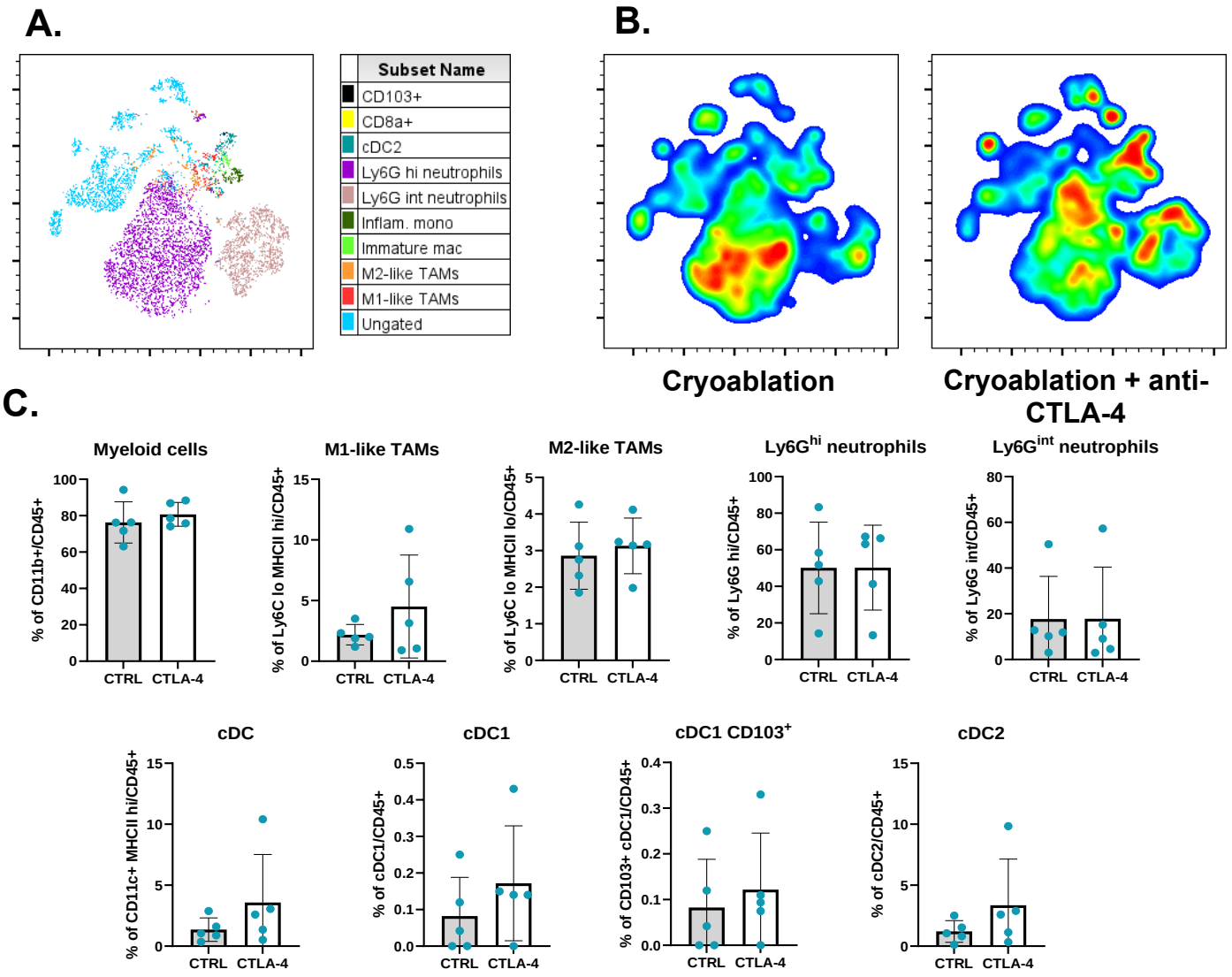
Supplementary figure 3. Comparison of ICOS$^+$ T cells expressing effector/effector memory subtype markers. Representative abscopal tumor dot plots gating on ICOS$^+$ CD4$^+$ (A) and CD8$^+$ (B) T cells; the different colors denote the different subpopulations of T cells, as indicated in the legend provided. E_EM = effector/effector memory; CM = central memory; CTRL = control; CTLA-4 = anti-CTLA-4; PD-L1 = anti-PD-L1; PD-1 = anti-PD-1.

Supplementary figure 4.



Supplementary figure 4. Effects of anti-CTLA-4 administration on lymphocyte infiltration of tumors. TIL score of primary and abscopal tumors. Blue bars represent controls and purple bars represent anti-CTLA-4 treatment. Unpaired Student's t-test for normally distributed data or Mann-Whitney U-test for non-normally distributed data was performed comparing naïve mice to mice treated with anti-CTLA-4 or cryoablation monotherapy to combination with anti-CTLA-4, with $p<0.05$ (*) considered significant. CTRL = control; CTLA-4 = anti- CTLA-4. n = 5 per group.

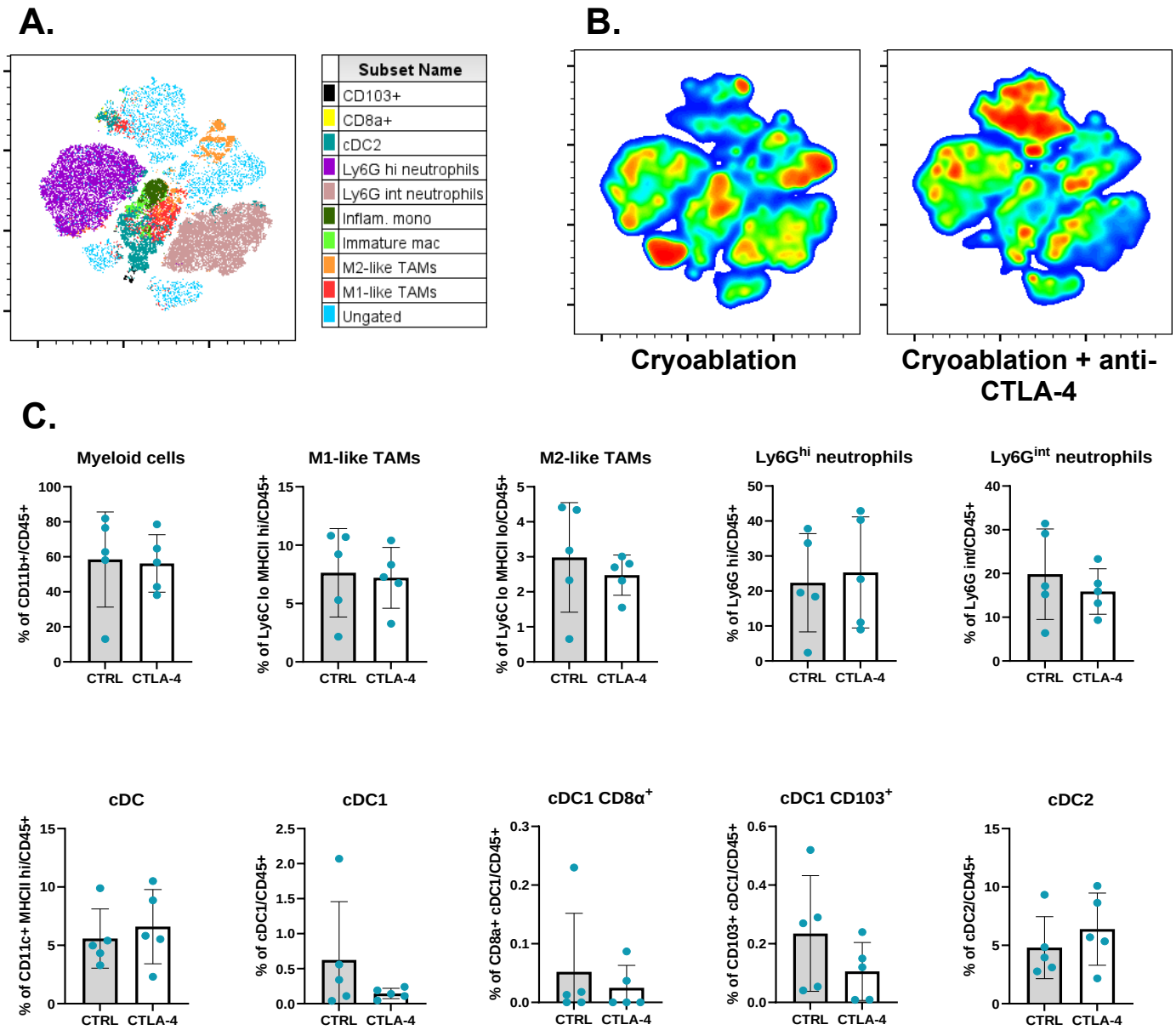
Supplementary figure 5.



Supplementary figure 5. Myeloid infiltration of primary tumors following CTLA-4 blockade combined with cryoablation. (A) Representative tSNE map from one experimental repeat, showing the clustering of different myeloid populations at the primary tumors based on flow cytometry parameters' similarity. (B) Representative tSNE heatmap from one experimental repeat, made from concatenated primary tumors from cryoablation monotherapy versus cryoablation plus anti-CTLA-4. (C) Myeloid populations,

analyzed as frequency of the live immune cells ($CD45^+$), at the primary tumors. Unpaired Student's t-test for normally distributed data or Mann-Whitney U-test for non-normally distributed data was performed comparing cryoablation monotherapy to combination with anti-CTLA-4, with $p<0.05$ considered significant. TAMs = tumor-associated macrophages; hi = high; int = intermediate; cDC = conventional dendritic cells; CTRL = control; CTLA-4 = anti-CTLA-4. n = 5 per group.

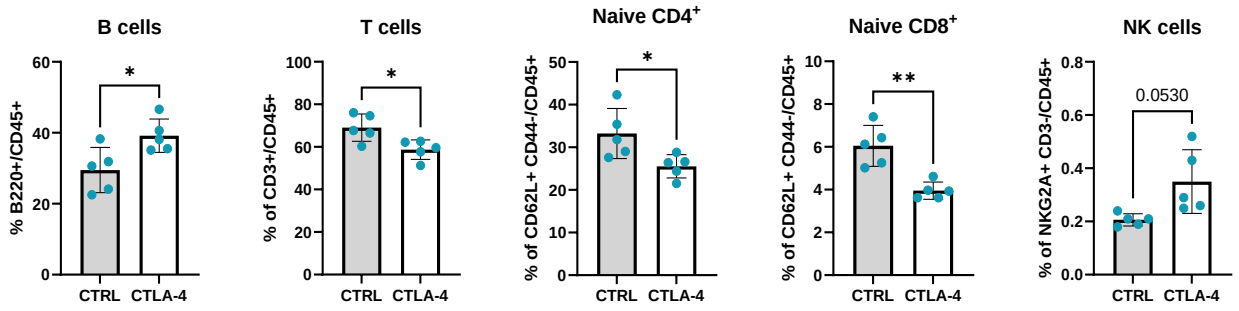
Supplementary figure 6.



Supplementary figure 6. Myeloid infiltration of abscopal tumors following CTLA-4 blockade combined with cryoablation. (A) Representative tSNE map from one experimental repeat, showing the clustering of different myeloid populations at the abscopal tumors based on flow cytometry parameters' similarity. (B) Representative tSNE

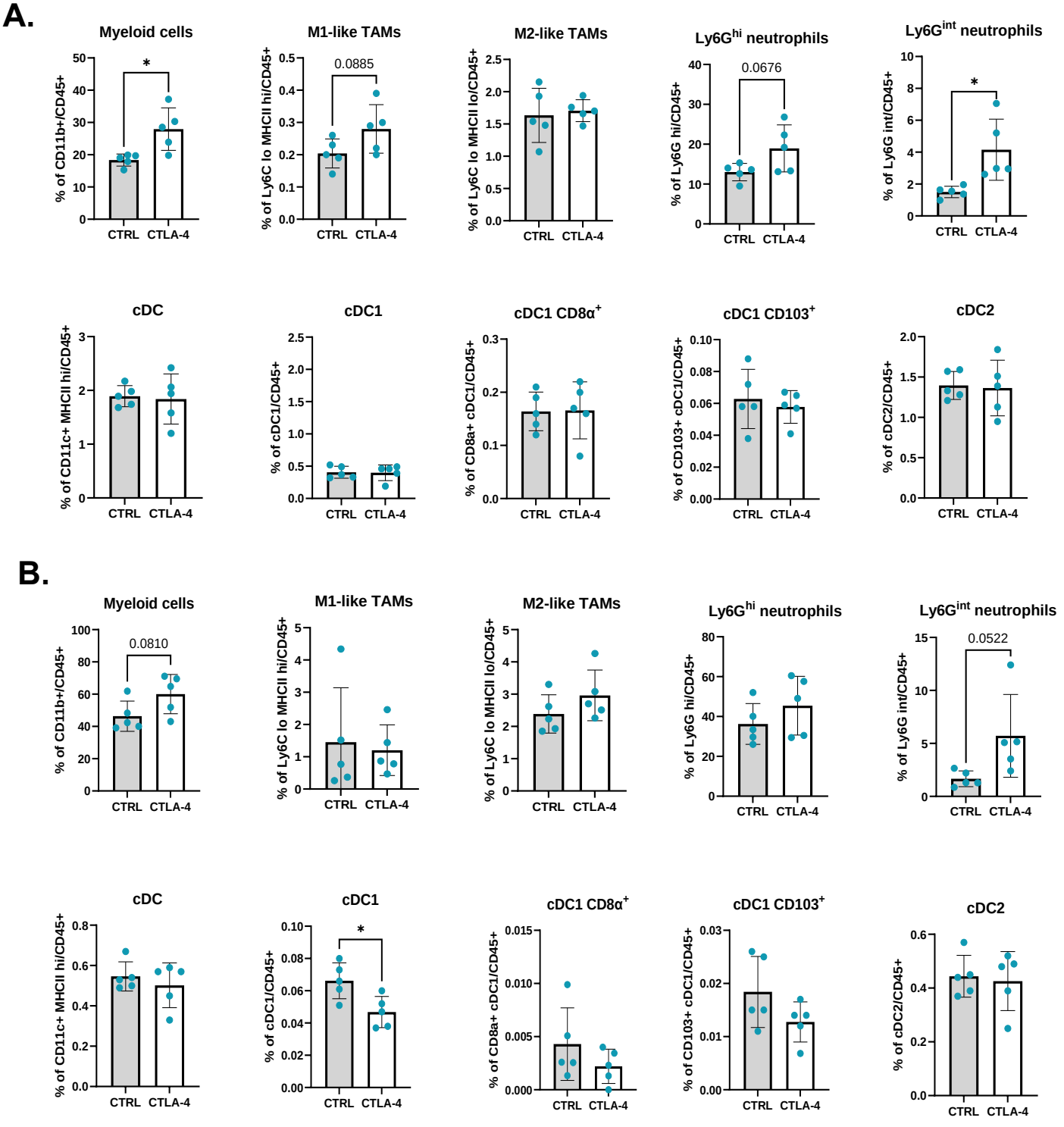
heatmap from one experimental repeat, made from concatenated abscopal tumors from cryoablation monotherapy versus cryoablation plus anti-CTLA-4. (C) Myeloid populations, analyzed as frequency of the live immune cells ($CD45^+$), at the abscopal tumors. Unpaired Student's t-test for normally distributed data or Mann-Whitney U-test for non-normally distributed data was performed comparing cryoablation monotherapy to combination with anti-CTLA-4, with $p<0.05$ considered significant. TAMs = tumor-associated macrophages; hi = high; int = intermediate; cDC = conventional dendritic cells; CTRL = control; CTLA-4 = anti-CTLA-4. n = 5 per group.

Supplementary figure 7.



Supplementary figure 7. Cryoablation plus CTLA-4 blockade decreases the proportion of naïve T cells at the primary TdLN. Parent immune populations and T cell subpopulations, analyzed as frequency of the live immune cells ($CD45^+$), at the primary TdLN. Unpaired Student's t-test for normally distributed data or Mann-Whitney test for non-normally distributed data was performed comparing cryoablation monotherapy to combination with anti-CTLA-4, with $p<0.05$ (*) and $p<0.01$ (**) considered significant; trends are shown where relevant. CTRL = control; CTLA-4 = anti-CTLA-4. n = 5 per group.

Supplementary figure 8.



Supplementary figure 8. Frequency of myeloid cells systemically. Myeloid cells, analyzed as frequency of the live immune cells ($CD45^+$), at the spleen (A) and peripheral blood (B). Unpaired Student's t-test for normally distributed data or Mann-Whitney U-test for non-normally distributed data was performed comparing cryoablation monotherapy to combination with anti-CTLA-4, with $p<0.05$ (*) considered significant. TAMs = tumor-associated macrophages; hi = high; int = intermediate; cDC = conventional dendritic cells; CTRL = control; CTLA-4 = anti-CTLA-4. n = 5.

Supplementary table 1. Antibodies used for flow cytometry

	Antibody	Clone	Fluorophore	Catalog number	Manufacturer	Concentration
Lymphocyte panel	NKG2A	20d5	APC	564383	BD	1:100
	CD3	17A2	APC-Cy7	100222	Biolegend	1:50
	CD45	30-F11	AF700	103128	Biolegend	1:100
	B220	RA3-6B2	BV421	103240	Biolegend	1:50
	ICOS	C396.4A	BV605	567920	BD	1:100
	CD8	53-6.7	BV650	100742	Biolegend	1:100
	CD107a	1D4B	BV711	564348	BD	1:200
	CD127	SB/199	BV786	563748	BD	1:200
	CD11b	M1/70	FITC	11-0112-85	eBioscience	1:200
	CD25	PC61	PE	12-0251-83	eBioscience	1:100
	CD62L	MEL-14	PE-CF594	562404	BD	1:200
	CD4	GK1.5	PE-Cy7	25-0041-82	Invitrogen	1:200
	CD44	IM7	PerCP-Cy5.5	560570	BD	1:50
	CCR3	J073E5	APC	144512	Biolegend	1:100
Myeloid Panel	CD8a	53-6.7	APC-Cy7	100714	Biolegend	1:100
	CD45	30-F11	AF700	103128	Biolegend	1:50
	XCR1	ZET	BV421	148216	Biolegend	1:100
	Ly6C	HK1.4	BV605	128036	Biolegend	1:200
	MHCII	M5/114.15.2	BV650	107641	Biolegend	1:100
	IL-5Ra	T21	BV711	740817	BD	1:200
	CD103	2E7	BV785	121439	Biolegend	1:100
	CD11b	M1/70	FITC	11-0112-85	eBioscience	1:200
	CD172a	P84	PE	144012	Biolegend	1:200
	Siglec-F	E-50-2440	PE-CF594	562757	BD	1:200
	CD11c	N418	PE-Cy7	117318	Biolegend	1:100
	Ly6G	1A8	PerCP-Cy5.5	560602	BD	1:50

Supplementary table 2. Immune phenotyping by flow cytometry

	Cell population	Expressed markers
Lymphocyte panel	B cells	$B220^{+}$
	Myeloid cells	$B220^{-}$ $CD3^{-}$ $CD11b^{+}$
	NK cells	$B220^{-}$ $CD3^{-}$ $NKG2A^{+}$
	T cells	$CD3^{+}$
	$CD4^{+}$ T cells	$CD3^{+}$ $CD4^{+}$
	$CD8^{+}$ T cells	$CD3^{+}$ $CD8^{+}$
	Activated T cells	$CD3^{+}$ $CD4^{+}$/$CD8^{+}$ $ICOS^{+}$
	Naïve T cells	$CD3^{+}$ $CD4^{+}$/$CD8^{+}$ $CD62L^{+}$ $CD44^{-}$
	Central memory T cells	$CD3^{+}$ $CD4^{+}$/$CD8^{+}$ $CD62L^{+}$ $CD44^{+}$
	Effector/effector memory cells	$CD3^{+}$ $CD4^{+}$/$CD8^{+}$ $CD62L^{-}$ $CD44^{+}$
	Pre-effector T cells	$CD3^{+}$ $CD4^{+}$/$CD8^{+}$ $CD62L^{-}$ $CD44^{-}$
	Regulatory T cells	$CD3^{+}$ $CD4^{+}$ $CD127^{low}$ $CD25^{+}$
Myeloid Panel	Conventional dendritic cells	$CD11c^{+}$ $CD11b^{low/+}$ $MHC\text{-}II^{high}$
	cDC1	$CD11c^{+}$ $CD11b^{low/+}$ $MHC\text{-}II^{high}$ $XCR1^{+}$
	Resident cDC1	$CD11c^{+}$ $CD11b^{low/+}$ $MHC\text{-}II^{high}$ $XCR1^{+}$ $CD8\alpha^{+}$
	Migratory cDC1	$CD11c^{+}$ $CD11b^{low/+}$ $MHC\text{-}II^{high}$ $XCR1^{+}$ $CD103^{+}$
	cDC2	$CD11c^{+}$ $CD11b^{low/+}$ $MHC\text{-}II^{high}$ $CD172^{+}$
	$Ly6G^{high}$ neutrophils	$CD11b^{+}$ $Ly6G^{high}$ $Siglec\text{-}F^{low}$
	$Ly6G^{intermediate}$ neutrophils	$CD11b^{+}$ $Ly6G^{intermediate}$ $Siglec\text{-}F^{high}$
	M1-like TAMs	$CD11b^{+}$ $Ly6C^{low}$ $MHC\text{-}II^{high}$
	M2-like-TAMs	$CD11b^{+}$ $Ly6C^{low}$ $MHC\text{-}II^{low}$
	Inflammatory monocytes	$CD11b^{+}$ $Ly6C^{high}$ $MHC\text{-}II^{low}$
	Immature macrophages	$CD11b^{+}$ $Ly6C^{+}$ $MHC\text{-}II^{high}$