Supplemental Tables

Supplemental Table S1.

Dose escalation scheme

Dose level	Evofosfamide IV (mg/m^2) over 60 mins on days 1 and 8 every 21 days × 2	Ipilimumab IV (mg/kg) over 90 mins on day 8 every 21 days × 4
-1	320	3
1 (starting level)	400	3
2	480	3
3	560	3
4	640	3

Supplemental Table S2.

Fifty most differentially expressed genes between responders and non-responders

Up/downregulated in Non-Responders Pre-Treatment				Up/downregulated in Non-Responders On-Treatment			
SPINK13	5.5	HLA-DRB5	-6.52	ALX1	8.38	DDC	-8.78
GSTM1	5.21	INSM1	-5.77	HOXC10	8.16	GPM6A	-7.61
CHST9	5.02	BTNL8	-4.61	SOX2	7.96	AC006449.	-7.47
AC016577.	4.86	FCGBP	-4.19	SLITRK2	7.55	ANKS4B	-6.7
TNNT1	4.66	FCER1A	-3.86	MAGEA3	7.23	ARID3C	-6.64
MAGEA1	4.56	CD1E	-3.86	MAGEA6	7.09	CXCL6	-6.37
CYP3A43	4.48	LINGO3	-3.85	XAGE1B	6.59	MPPED1	-6.2
MEPE	4.48	SOD3	-3.74	CSAG1	6.55	MROH2A	-5.92
AARD	4.34	FAM159A	-3.71	HOXD8	6.53	KNDC1	-5.68
RPRML	4.27	C19orf35	-3.35	KRT75	6.5	CLDN14	-5.52
LY6K	4.02	NLGN4Y	-3.34	XAGE1A	6.44	CFTR	-5.24
C1QTNF9B	3.97	CLEC17A	-3.25	MCIDAS	6.28	HLF	-5.23
MAPK10	3.93	SLC34A3	-3.1	EN1	6.13	PDIA2	-5.08
SYT16	3.78	RHOH	-3.05	CHL1	5.97	ANKRD30B	-4.84
NME5	3.74	ADGRL3	-2.78	FBLL1	5.84	NRG1	-4.8
CES3	3.62	LFNG	-2.74	CXorf49B	5.68	ASIC2	-4.59
CCNO	3.54	TBXAS1	-2.73	CXorf49	5.68	RELN	-4.4
A2ML1	3.44	TMEM163	-2.7	SHOX2	5.5	KANK4	-4.34
SFTA3	3.36	P2RY14	-2.52	HOXD4	5.4	NDST3	-4.31
GLOD5	3.36	FMOD	-2.5	SOSTDC1	5.35	MYOM2	-4.22
DDX25	3.36	CD38	-2.48	HBM	5.23	NCAM2	-4.21
LRP2	3.3	RNF224	-2.42	BMP7	4.92	SLC16A10	-4.14
ROPN1B	3.27	ADRA2A	-2.37	HOXC9	4.75	DCDC2	-4.07
MAPK15	3.24	C16orf54	-2.32	DCLK1	4.36	MAB21L3	-4.02
KAZN	3.16	LY86	-2.31	NSG1	4.3	USH2A	-3.99
ABCC12	3.13	PAQR8	-2.16	A2ML1	4.21	HPGD	-3.98
GPR37	3.05	PTPRCAP	-2.02	TTC39A	4.09	SLC19A3	-3.95
HRSP12	2.78	FERMT3	-1.99	GJC1	4.02	CFAP57	-3.94
VSTM2L	2.54	MEGF6	-1.87	ANK2	3.86	KLHL13	-3.84
SLC13A3	2.54	MTRNR2L8	-1.82	LINGO1	3.84	LRRC4C	-3.28
SLC7A2	2.35	ACKR4	-1.81	BUB1	3.69	NMNAT3	-3.23
SPECC1	2.29	PM20D1	-1.74	CDKN3	3.52	AKR1C3	-3.23
ZNF662	2.12	SMPD3	-1.73	WNK4	3.51	PRKAA2	-3.22
CHCHD6	2	GLIPR2	-1.67	NELL2	3.48	KAAG1	-3.15
MTFR1	1.99	SEMA4A	-1.66	AIF1L	3.42	MAGI2	-3.1
FSBP	1.99	LAG3	-1.63	SAMD3	3.38	RPH3AL	-3.09
PHLPP1	1.96	CXCR4	-1.59	CXCL9	3.38	KLHL31	-2.99
BNIPL	1.95	MZT2A	-1.56	SLC12A5	3.34	GLTPD2	-2.85
TSPAN6	1.94	ZNF671	-1.54	EBF1	3.34	ZNF140	-2.81
MIPEP	1.93	C5orf56	-1.54	TWIST1	3.3	ST20	-2.58
PSAT1	1.9	GATA6	-1.46	ARHGAP11	3.24	FBP1	-2.48
SNX25	1.89	ICOSLG	-1.43	TCF7L1	3.11	MPST	-2.25
TBX6	1.84	GMIP	-1.42	LZTS1	3.09	ACAA2	-2.24
ADHFE1	1.81	CBX7	-1.41	ASB2	3.08	OVGP1	-2.18
HPRT1	1.8	IRF7	-1.38	FAM107A	3.06	NPB	-2.16
TMEM99	1.76	KIF21B	-1.38	CXorf36	3.01	SERINC2	-2.14
FBXO32	1.7	DGKZ	-1.37	FABP4	2.99	AIFM2	-2.13
GCAT	1.7	ZC3H12A	-1.33	HOPX	2.93	LYNX1	-2.11
RHOT1	1.68	PCDHB4	-1.33	KIF23	2.75	PECR	-2.11
ZNF572	1.67	CH507-9B2	-1.32	CDC45	2.74	CCBL1	-2.07

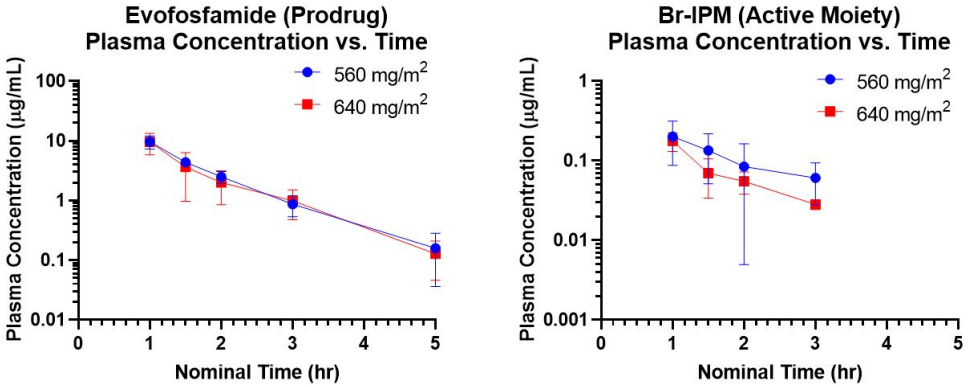
Supplemental Figures

Supplemental Figure S1

TH-CR-417 PK parameters: no significant difference between the two highest dose groups

	N	C_{max} (µg/mL)	T_{max} (hr)	AUC_{last} (hr*µg/mL)	AUC_{inf} (hr*µg/mL)	$T_{½}$ (hr)
Evofosfamide at 560 mg/m²						
Evofosfamide (Prodrug)	7	9.40	1	11.9	12.1	0.725
Br-IPM (Active Moiety)	7	0.179	1	0.228	NC	NC
Evofosfamide at 640 mg/m²						
Evofosfamide (Prodrug)	5	8.87	1	10.6	10.7	0.729
Br-IPM (Active Moiety)	4	0.182	1	0.203	NC	NC

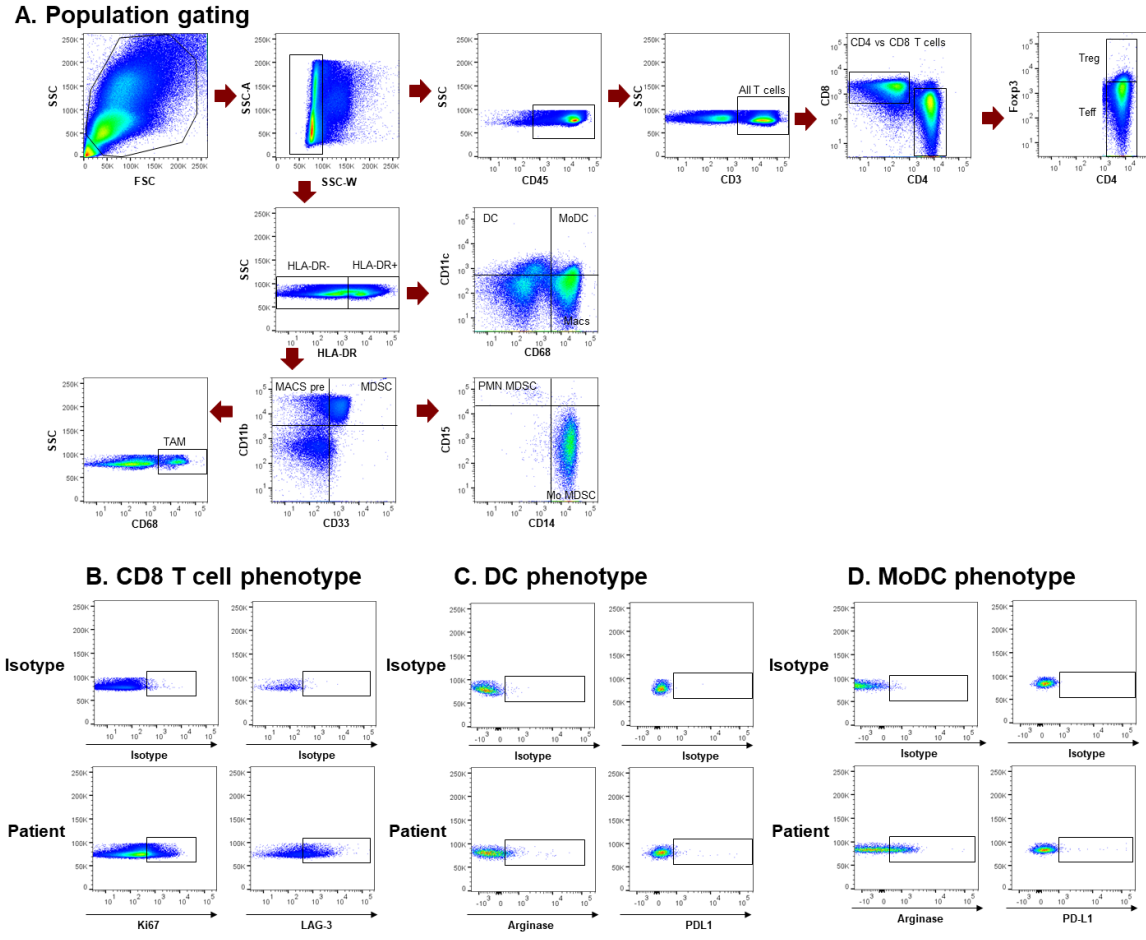
* Data presented are geometric means



Supplemental Figure S1.

Evofosfamide serum pharmacokinetics. Serial blood samples were collected from patients receiving evofosfamide at either 560 mg/m^2 or 640 mg/m^2 at the time points shown and analyzed for concentration of evofosfamide, the prodrug, and of bromo-isophosphoramide mustard (Br-IPM), the active moiety.

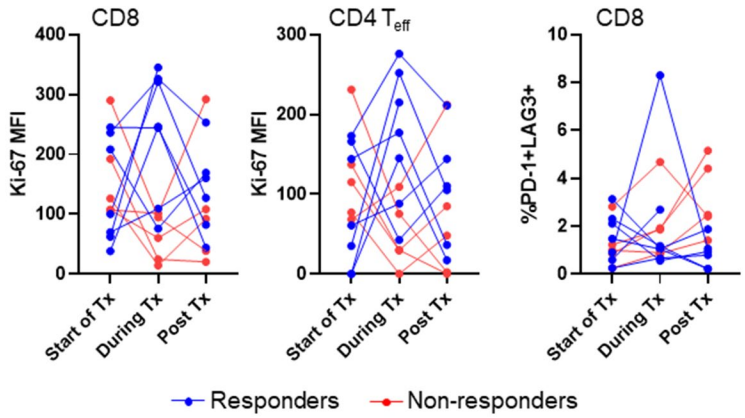
Supplemental Figure S2



Supplemental Figure S2.

Flow cytometry gating scheme for peripheral blood mononuclear cells. A) Debris and doublets were eliminated. $CD3^+$ T cells were subdivided into $CD8^+CD4^-$ cytotoxic T cells and $CD4^+CD8^-$ T cells. which were then subdivided into $CD4^+FOXP3^+$ T_{reg} and $CD4^+FOXP3^-$ T_{eff}. Dendritic cells were identified as $HLA^-DR^+CD11c^+CD68^-$ while monocytic dendritic cells were $HLA^-DR^+CD11c^+CD68^+$. Myeloid-derived suppressor cells (MDSC) were defined as $HLA^-DR^-CD11b^+CD33^+$ and further subdivided into PMN MDSC ($CD14^-CD15^+$), Mo MDSC ($CD15^-CD14^+$), and DN tumor-associated macrophages ($CD14^-CD15^-$). Tumor-associated macrophages were identified as $HLA^-DR^-CD11b^+CD33^-CD68^+$. **B-D)** Representative flow plots showing **B)** Ki67 (left) and Lag3 (right) staining in peripheral blood $CD8^+$ T cells, **C)** arginase 1 (left) and PD-L1 (right) staining in circulating dendritic cells, and **D)** Arginase 1 (left) and PD-L1 (right) staining in circulating monocytic dendritic cells relative to isotype control populations.

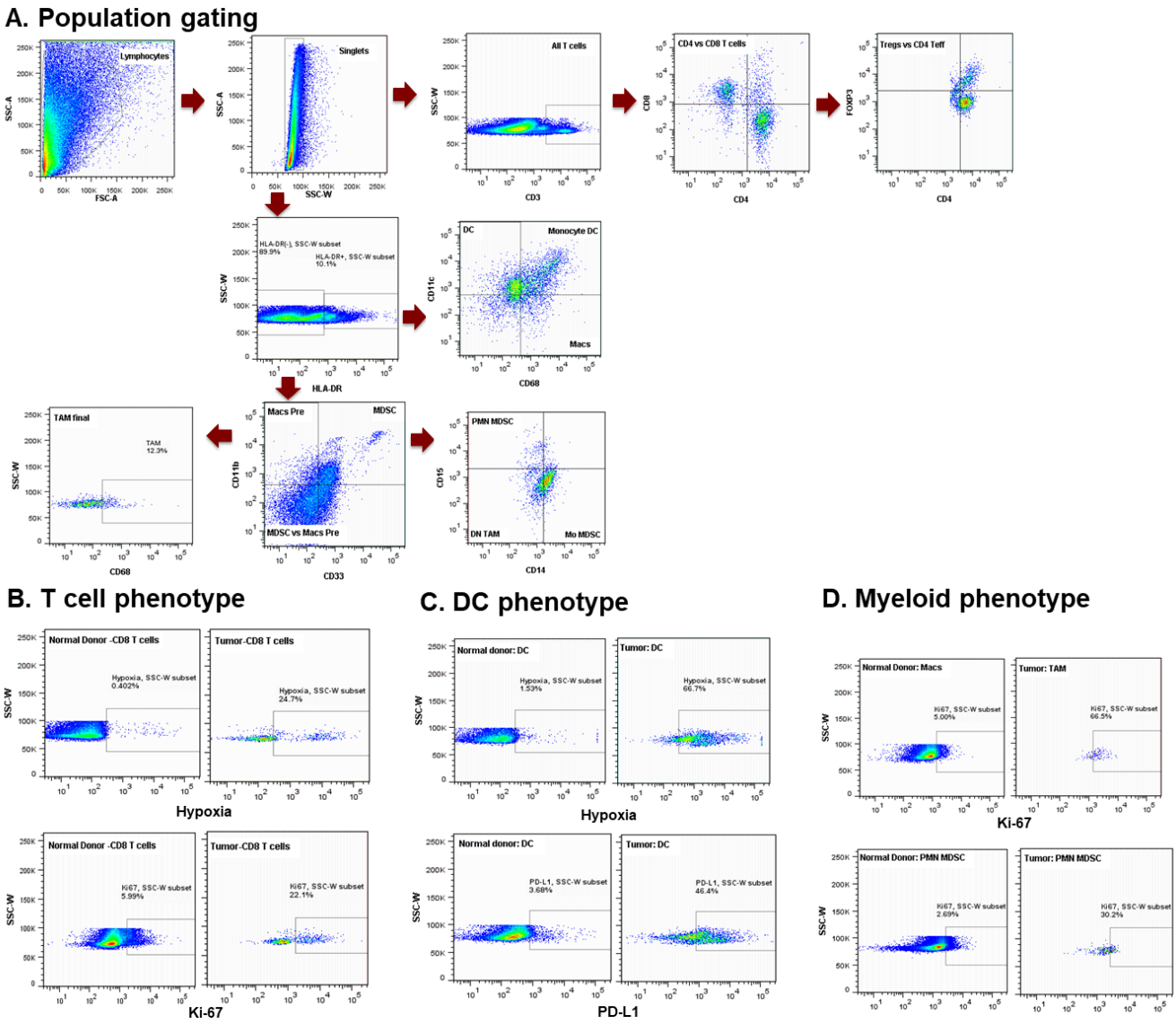
Supplemental Figure S3



Supplemental Figure S3.

Changes in circulating T cell phenotype due to treatment. Peripheral blood mononuclear cells were isolated from patients prior to, during, and post treatment and assessed for immune composition and function by 20-color flow cytometry. Proliferation of CD8 and CD4 effector T cells was analyzed by Ki-67 expression and is shown at each time point for each patient. The percentage of exhausted PD-1^{+}LAG-3^{+} CD8 T cells is also shown.

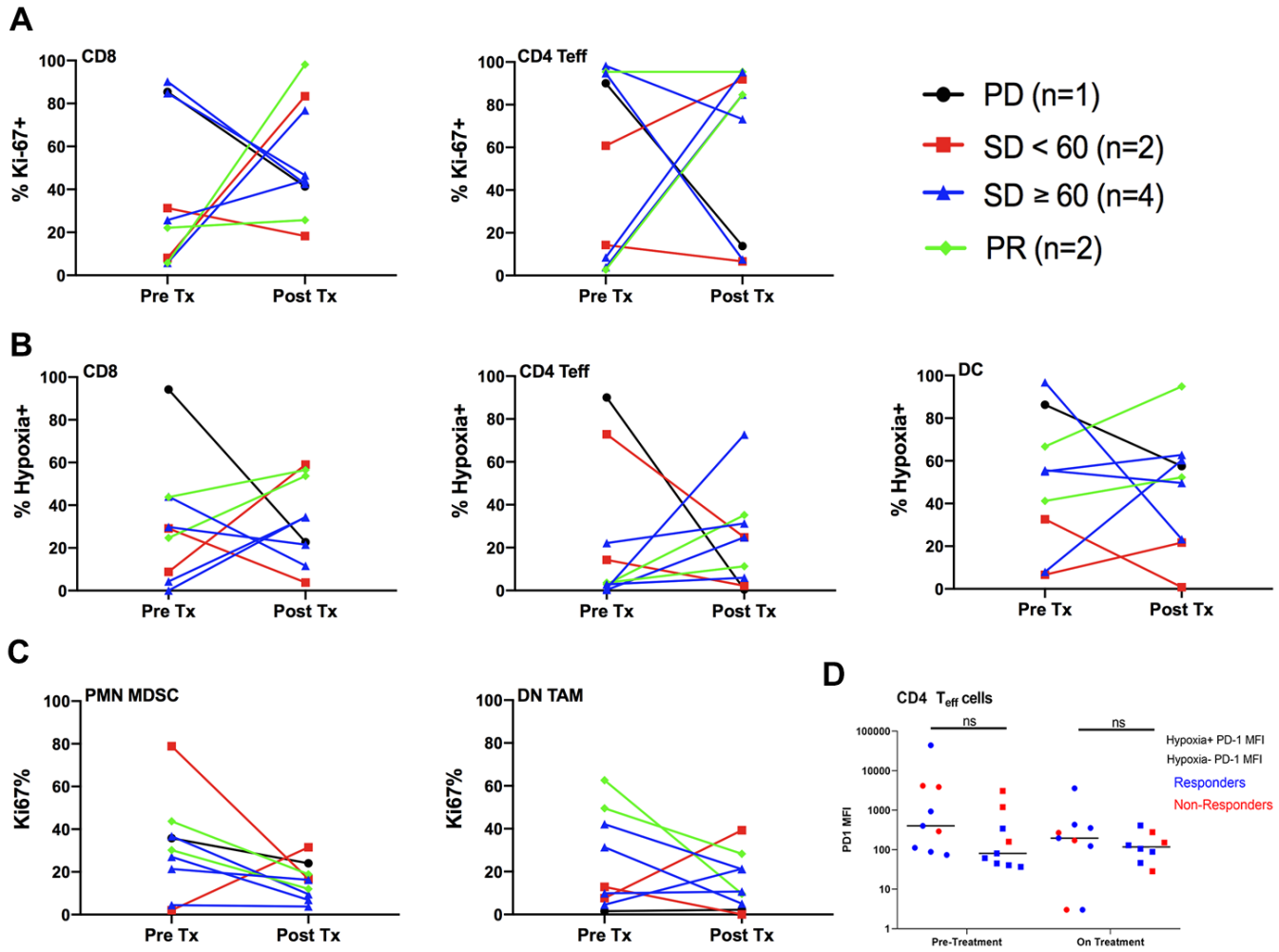
Supplemental Figure S4



Supplemental Figure S4.

Flow cytometry gating scheme for tumor biopsies. A) Debris and doublets were eliminated. $CD3^+$ T cells were subdivided into $CD8^+CD4^-$ cytotoxic T cells and $CD4^+CD8^-$ T cells, which were then subdivided into $CD4^+FOXP3^+$ T_{reg} and $CD4^+FOXP3^-$ T_{eff}. Dendritic cells were identified as $HLA^-DR^+CD11c^+CD68^-$ while monocytic dendritic cells were $HLA^-DR^+CD11c^+CD68^+$. Myeloid-derived suppressor cells (MDSC) were defined as $HLA^-DR^-CD11b^+CD33^+$ and further subdivided into PMN MDSC ($CD14^-CD15^+$), Mo MDSC ($CD15^-CD14^+$), and DN tumor-associated macrophages ($CD14^-CD15^-$). Tumor-associated macrophages were identified as $HLA^-DR^-CD11b^+CD33^-CD68^+$. **B-D)** Representative flow plots showing **B)** hypoxia (top) and Ki-67 (bottom) staining in tumor-infiltrating $CD8^+$ T cells, **C)** hypoxia (top) and PD-L1 (bottom) staining in tumor-infiltrating dendritic cells, and **D)** Ki-67 staining in tumor-associated macrophages (top) and PMN MDSC (bottom) relative to control populations in peripheral blood mononuclear cells from a healthy donor.

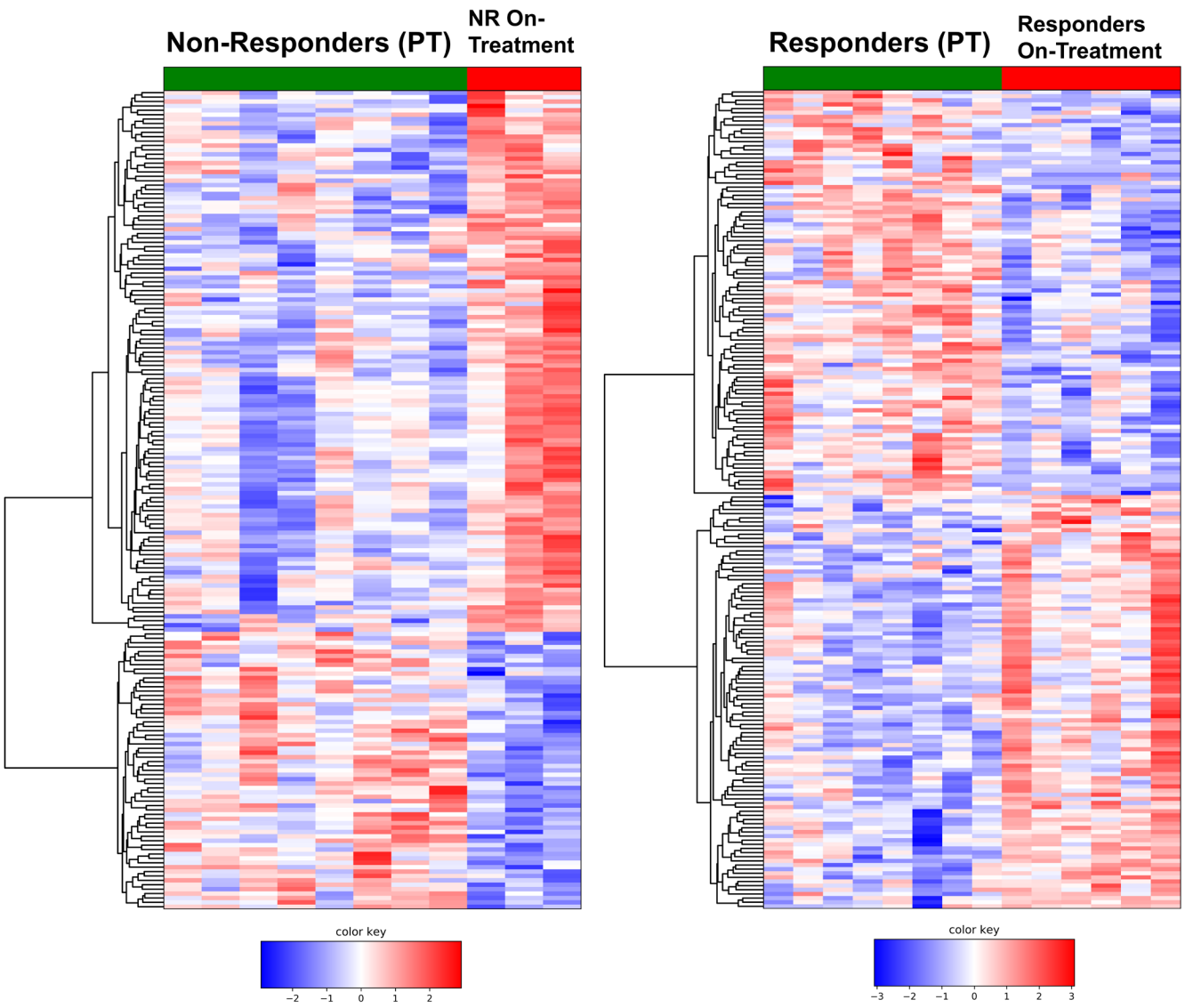
Supplemental Figure S5



Supplemental Figure S5.

Tumor hypoxia and immune function in individual patients. Patient biopsies were obtained at baseline and at week 7, and assessed for hypoxia and immune composition and function by 20-color flow cytometry. Data are shown for individual patients, color-coded based on response. **A)** Proliferation of tumor-infiltrating CD8 and CD4 effector T cells was analyzed based on Ki-67 expression. **B)** Patients were given oral pimonidazole 5-24 hours prior to biopsy to evaluate tumor hypoxia using anti-pimonidazole antibody conjugated to the fluorescent dye ATT0 594 (Hypoxyprobe), and densities of T cells and dendritic cells in hypoxic areas were assessed. **C)** Proliferation of immune-suppressive $CD33^{+}CD14^{-}CD15^{-}$ tumor-associated macrophages was evaluated by Ki-67 expression. D) Hypoxia-exposed (pimonidazole+, circles) vs non-hypoxia resident (pimonidazole-, squares) CD4 effector T cells were analyzed for PD-1 expression pre- and on-treatment (cycle 3, day 8).

Supplemental Figure S6



Supplemental Figure S6.

Changes in gene expression in response to evofosfamide + ipilimumab therapy. Tumor biopsies were collected pre-treatment and on-treatment (cycle 3, day 8), and RNA was isolated using All-prep (Qiagen). RNA sequencing was performed by Avera and bioinformatic analysis was performed by the Baylor College of Medicine Multi-omics Core.