

Supplementary Appendix

Supplement to: Taylor M, Betts CB, Maloney L, Nadler E, Algazi A, Guarino MJ, et al. Safety and Efficacy of Pembrolizumab in Combination With Acalabrutinib in Advanced Head and Neck Squamous Cell Carcinoma: Phase 2 Proof-of-Concept Study.

SUPPLEMENTAL METHODS

Dose-limiting Toxicities (DLT)

A DLT was defined as the occurrence of any of the following study drug–related adverse events (AEs):

- Grade 4 vomiting or diarrhea
- Grade 3 nausea, vomiting, or diarrhea lasting for over 72 hours
- Other grade ≥ 3 toxicities (transient grade 3–4 laboratory abnormalities that were not clinically significant were not considered DLTs)
- Dosing delay due to toxicity for more than 21 consecutive days

AEs clearly related to disease progression or the subject's current medical history and associated comorbidities were not considered DLTs.

Serious AEs

An AE was considered serious if it met one of the following criteria:

- It resulted in death
- It was life-threatening
- It required or prolonged hospitalization
- It resulted in persistent or significant disability or incapacity
- It resulted in a congenital anomaly or birth defect in a neonate/infant born to a mother exposed to the study drug
- It was considered a significant medical event by the investigator based on medical judgment

SUPPLEMENTAL TABLES

Table S1. Antibody Panels Used in mIHC for Assessment of Tumor Immune Microenvironment. Antibody panels focused on lymphoid, myeloid, and functional assessment were utilized in a cyclic immunohistochemistry platform. AEC, aminoethyl carbazole; O/N, overnight; ms, mouse; rbt, rabbit; RT, room temperature.

Lymphoid Panel							
Cycle	Primary antibody	Clone/ Product #	Dilution	Reaction	Histofine	Reaction	AEC
1	Hematoxylin	Dako	Original	1 min			
2	Ms anti PD1	NAT105	1/50	RT, 30 min	Anti-mouse	RT, 30 min	20 min
3	Rbt anti CD3	SP7	1/150	RT, 30 min	Anti-rabbit	RT, 30 min	20 min
4	Ms anti RORyt	6F3.1	1/200	RT, 30 min	Anti-mouse	RT, 30 min	10 min
5	Ms anti NKp46	195314	1/20	4°C, O/N	Anti-mouse	RT, 30 min	20 min
6	Ms anti CD8	C8/144B	1/100	RT, 30 min	Anti-mouse	RT, 30 min	20 min
7	Rbt anti T-bet	sc-21003	1/100	RT, 30 min	Anti-rabbit	RT, 30 min	20 min
8	Ms anti GATA-3	L50-823	1/100	RT, 30 min	Anti-mouse	RT, 30 min	40 min
9	Rbt anti PDL1	E1L3N	1/100	RT, 60 min	Anti-rabbit	RT, 30 min	40 min
10	Ms anti CD45	H130	1/100	RT, 30 min	Anti-mouse	RT, 30 min	20 min
11	Ms anti Foxp3	236A/E7	1/40	RT, 30 min	Anti-mouse	RT, 30 min	20 min
12	Ms anti CD20	O.N.85	1/1000	RT, 30 min	Anti-mouse	RT, 30 min	20 min
13	Rbt anti BTK	D3H5	1/100	RT, 30 min	Anti-rabbit	RT, 30 min	30 min
14	Ms anti p16	E6H4	Prediluted	RT, 30 min	Anti-mouse	RT, 30 min	20 min
Myeloid Panel							
Cycle	Primary antibody	Clone/ Product #	Dilution	Reaction	Histofine	Reaction	AEC
1	Hematoxylin	Dako	Original	1 min			
2	Rbt anti-CSF1R	SP211	1/150	RT, 30 min	Anti-rabbit	RT, 30 min	20 min
3	Ms anti PD1	NAT105	1/50	RT, 30 min	Anti-mouse	RT, 30 min	20 min
4	Ms anti CD68	PG-M1	1/50	RT, 30 min	Anti-mouse	RT, 30 min	20 min
5	Ms anti DC-SIGN	DC-28	1/100	RT, 30 min	Anti-mouse	RT, 30 min	20 min
6	Rat anti DC-LAMP	1010E1.01	1/100	RT, 30 min	Anti-rat	RT, 30 min	30 min
7	Ms anti CD66b	G10F5	1/600	RT, 30 min	Anti-mouse	RT, 30 min	20 min
8	Rbt anti MHC II	EPR11226	1/5000	RT, 30 min	Anti-rabbit	RT, 30 min	20 min
9	Ms anti CD163	10D6	1/100	RT, 30 min	Anti-mouse	RT, 30 min	20 min
10	Rbt anti PDL1	E1L3N	1/100	4°C, O/N	Anti-rabbit	RT, 30 min	40 min
11	Ms anti CD45	H130	1/100	RT, 30 min	Anti-mouse	RT, 30 min	20 min
12	CD3/20/NKp46*	*	*	RT, 30 min	**	RT, 30 min	20 min
13	Ms anti Tryptase	AA1	1/20,000	RT, 30 min	Anti-mouse	RT, 30 min	20 min
14	Rbt anti BTK	D3H5	1/100	RT, 30 min	Anti-rabbit	RT, 30 min	30 min
15	Ms anti p16	E6H4	Prediluted	RT, 30 min	Anti-mouse	RT, 30 min	20 min
Functional Panel							
Cycle	Primary antibody	Clone/ Product #	Dilution	Reaction	Histofine	Reaction	AEC
1	Hematoxylin	Dako	Original	1 min			
2	Ms anti CD4	4B12	1/25	RT, 30 min	Anti-mouse	RT, 30 min	40 min
3	Rbt anti CD3	SP7	1/150	RT, 30 min	Anti-rabbit	RT, 30 min	20 min
4	Ms anti PD1	NAT105	1/50	RT, 30 min	Anti-mouse	RT, 30 min	40 min
5	Rbt anti Ki67	SP6	1/500	RT, 30 min	Anti-rabbit	RT, 30 min	20 min
6	Ms anti CD8	C8/144B	1/50	RT, 30 min	Anti-mouse	RT, 30 min	20 min
7	Ms anti CD45	H130	1/50	RT, 30 min	Anti-mouse	RT, 30 min	40 min
8	Rbt anti EOMES	AB2283	1/1000	RT, 30 min	Anti-rabbit	RT, 30 min	20 min
9	Rbt anti ICOS	SP98	1/100	RT, 30 min	Anti-rabbit	RT, 30 min	40 min
10	Ms anti CD68	PG-M1	1/50	RT, 30 min	Anti-mouse	RT, 30 min	20 min
11	Rbt anti Granzyme B	EP230	1/100	RT, 30 min	Anti-rabbit	RT, 30 min	20 min

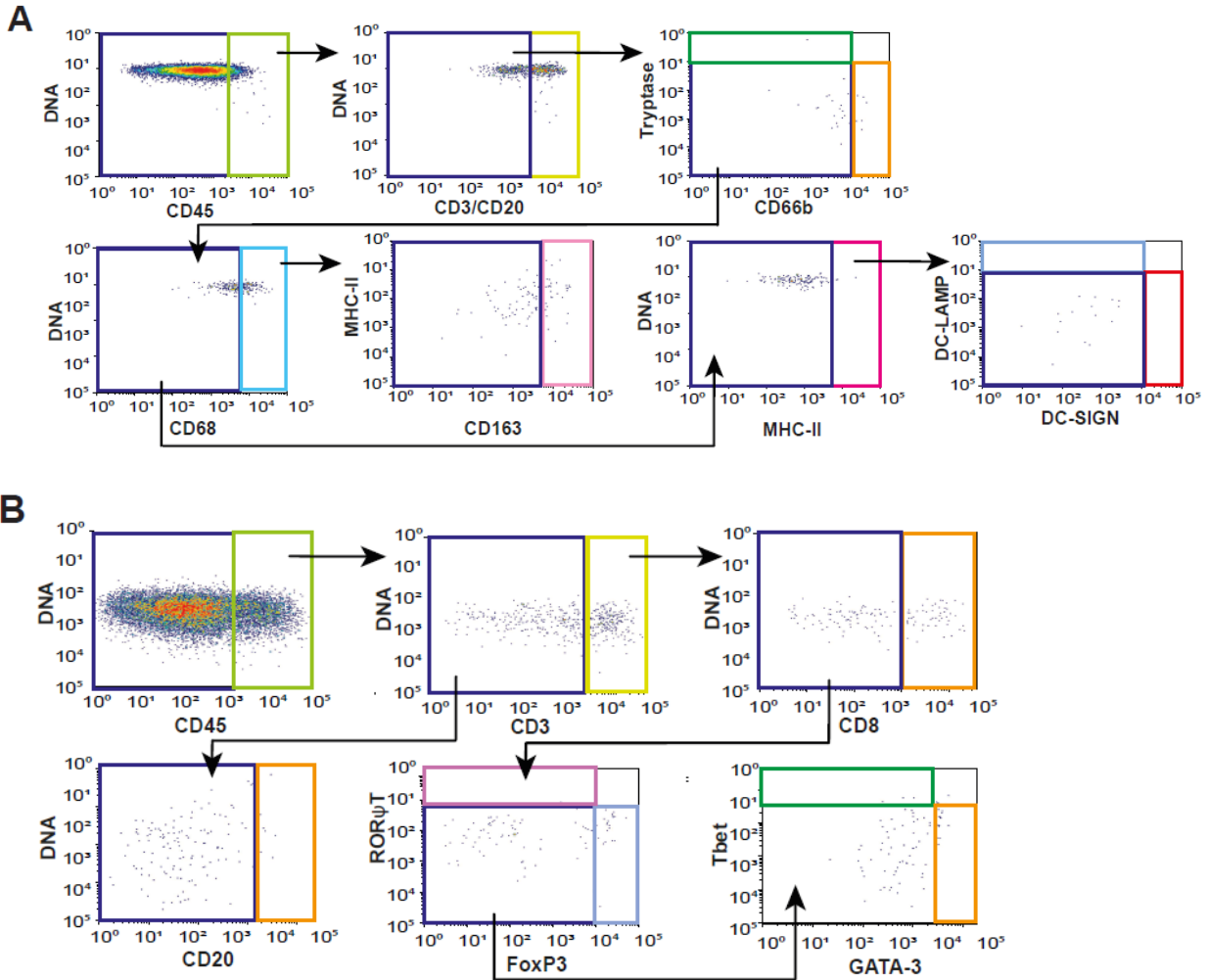
*CD3(SP7):1/150, CD20(O.N.85):1/1,000, NKp46(195314):1/20.
 **Anti-mouse for 30 min & anti-rabbit for 30 min.

Table S2. Tissue Area Assessed in Baseline and On-Treatment Patient Biopsies by Multiplex Immunohistochemistry. On-treatment biopsies were collected 43 days after starting treatment. Cohort averages are reported in the bottom row. Patients P1, P3, P5, P6, and P9 received pembrolizumab monotherapy. Patients P2, P4, P7, and P10 received pembrolizumab plus acalabrutinib combination therapy. Rx, on treatment.

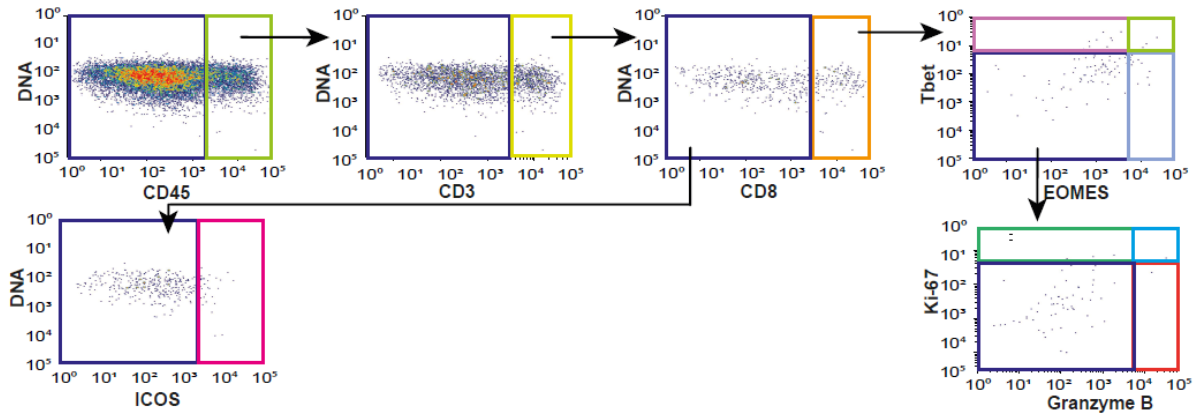
Patient ID	Biopsy type	Total area assessed	% biopsy tissue assessed
P1	Baseline	17775273 μm^2	35.2%
	Rx	3212457 μm^2	40.0%
P2	Baseline	93871509 μm^2	22.4%
	Rx	8234708 μm^2	40.5%
P3	Baseline	4055441 μm^2	12.9%
	Rx	594248 μm^2	16.8%
P4	Baseline	464850 μm^2	61.7%
	Rx	615190 μm^2	100%
P5	Baseline	2215000 μm^2	67.9%
	Rx	1857157 μm^2	15.1%
P6	Baseline	7865899 μm^2	69.0%
	Rx	4642918 μm^2	70.1%
P7	Baseline	5130340 μm^2	67.5%
	Rx	-	-
P9	Baseline	2927323 μm^2	55.4%
	Rx	7990285 μm^2	90.3%
P10	Baseline	1105928 μm^2	100%
	Rx	-	-
COHORT AVERAGES:		10,159,568 μm^2 (10.16 mm^2)	51.2%

SUPPLEMENTAL FIGURES

Figure S1. Hierarchical Image Cytometry Gating Strategies Using Multiplex Immunohistochemistry for Quantification of Immune Cell Populations. (A) myeloid antibody panel, (B) lymphoid antibody panel, and (C) functional antibody panel gating strategies. (D) Immune cell populations identified via gating strategies shown.



C



D

Lineage	Identification
Th1 helper T cells	CD45 ⁺ CD3 ⁺ CD8 ⁻ Tbet ⁺
Th2 helper T cells	CD45 ⁺ CD3 ⁺ CD4 ⁺ CD8 ⁻ GATA3 ⁺
Regulatory T cells (Treg)	CD45 ⁺ CD3 ⁺ CD4 ⁺ CD8 ⁻ Foxp3 ⁺
Th17 helper T cells	CD45 ⁺ CD3 ⁺ CD4 ⁺ CD8 ⁻ RORγt ⁺
Th0 (naïve) helper T cells	CD45 ⁺ CD3 ⁺ CD4 ⁺ CD8 ⁻ Foxp3 ⁻ RORγt ⁻ Tbet ⁻ GATA3 ⁻
CD8 ⁺ T lymphocytes	CD45 ⁺ CD3 ⁺ CD8 ⁺
CD20 ⁺ B cells	CD45 ⁺ CD3 ⁻ CD20 ⁺
Granulocytes	CD45 ⁺ CD3/CD20 ⁻ CD66b ⁺
Mast cells	CD45 ⁺ CD3/CD20 ⁻ CD66b ⁻ Tryptase ⁺
Macrophages+Monocytes	CD45 ⁺ CD3/CD20 ⁻ CD66b ⁻ Tryptase ⁻ CD68 ⁺ CD163 ^{+/-}
Immature DC	CD45 ⁺ CD3/CD20 ⁻ CD66b ⁻ Tryptase ⁻ CD68 ⁻ MHC-II ⁺ DC-LAMP ⁻ DC-SIGN ^{-/+}
Mature DC	CD45 ⁺ CD3/CD20 ⁻ CD66b ⁻ Tryptase ⁻ CD68 ⁻ MHC-II ⁺ DC-LAMP ⁺
CD45 ⁺ other	CD45 ⁺ CD3/CD20 ⁻ CD66b ⁻ Tryptase ⁻ CD68 ⁻ MHC-II ⁻
Proliferation	Ki67
Cytotoxicity	Granzyme B
Immune functionality	EOMES, Tbet, ICOS

Figure S2. Patient Disposition.

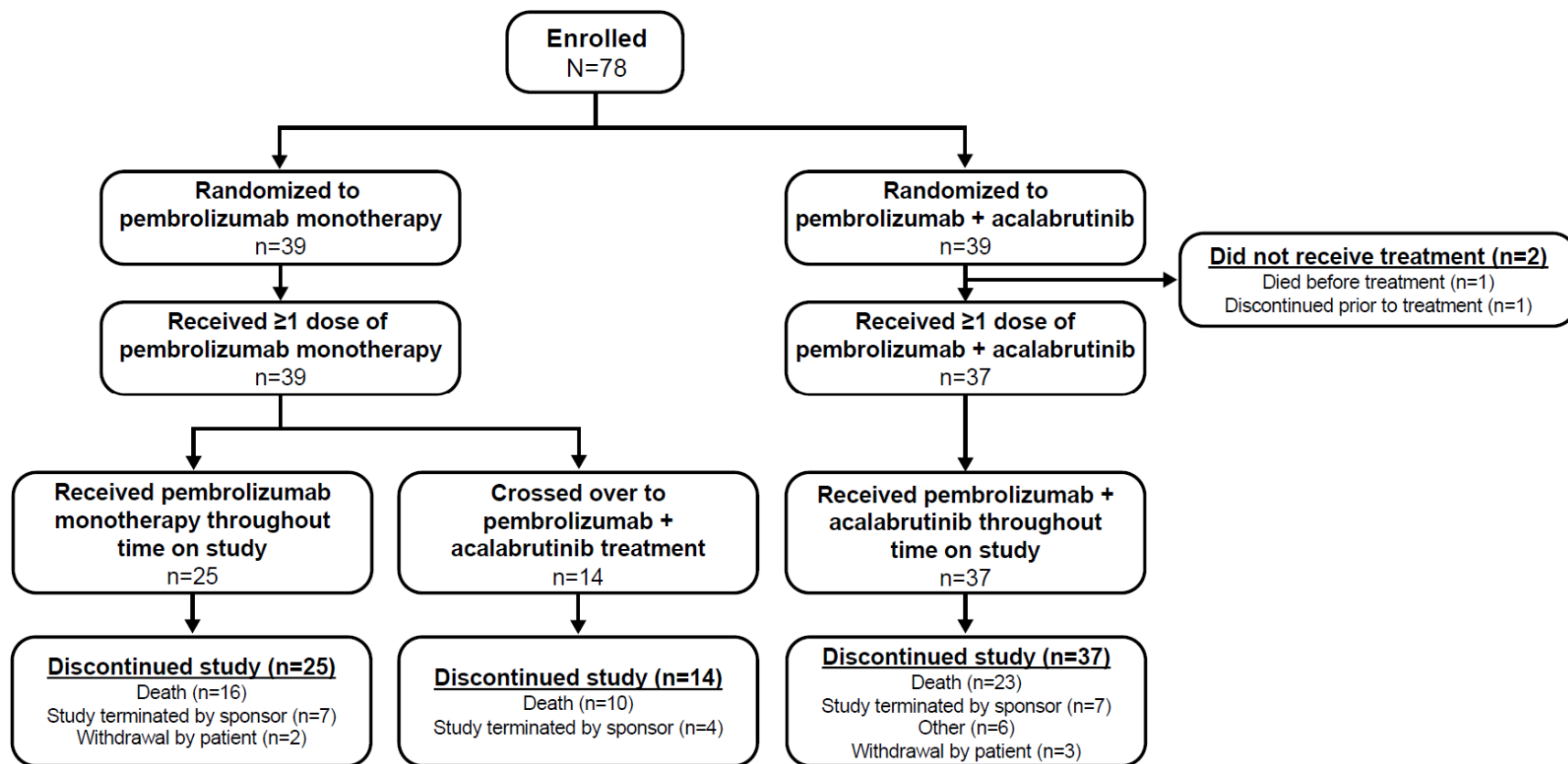
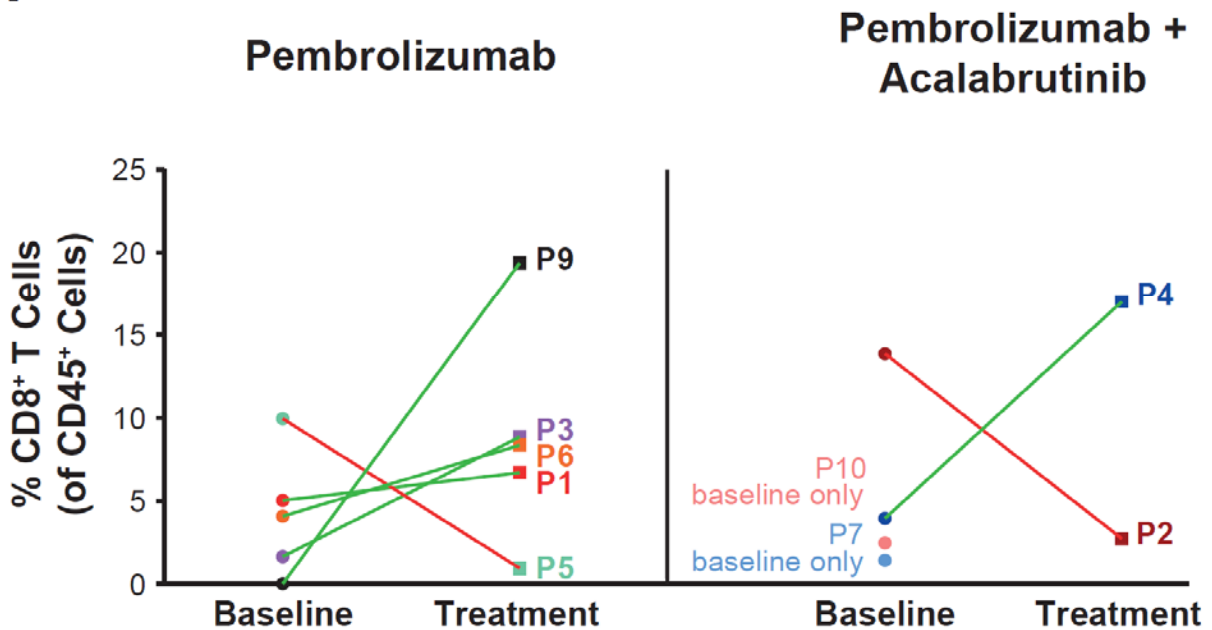


Figure S3. TiME at Baseline and After Treatment.

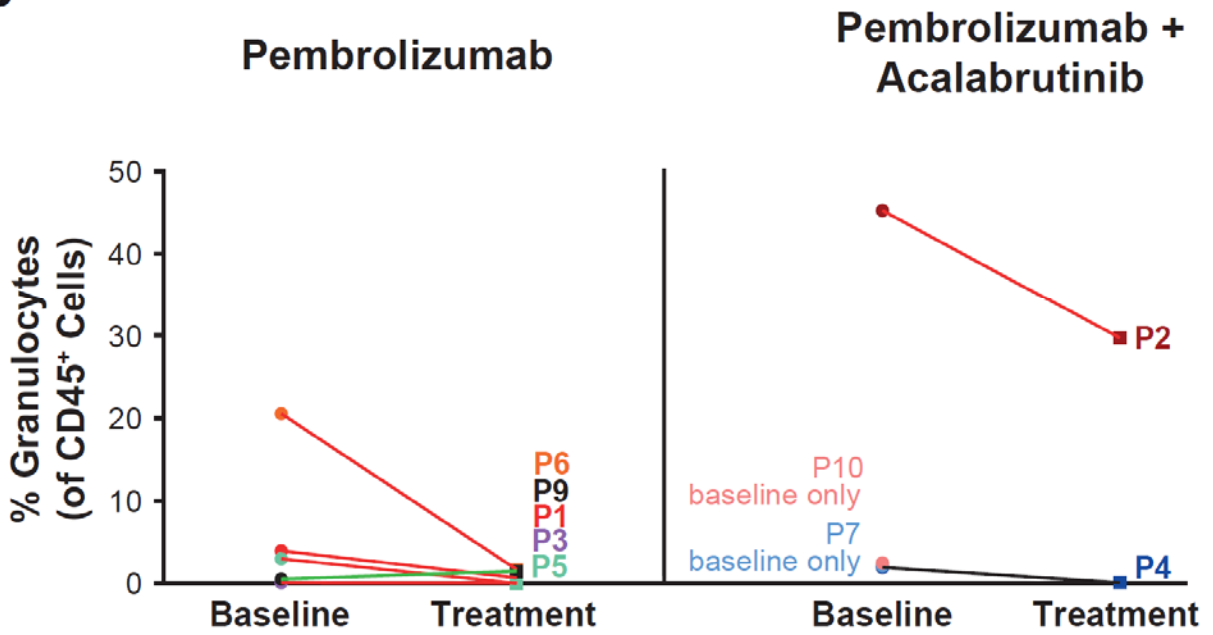
(A) Percentages of total CD45 cells that were CD8⁺, (B) percentages of total CD45⁺ cells that were granulocytes, (C) ratios of CD163⁻ to CD163⁺CD68⁺ monocytes/macrophages, and (D) percentages of total CD4⁺ T cells that were positive for ICOS are shown for each patient at baseline and after 43 days of treatment.

Pembrolizumab monotherapy, n=5; pembrolizumab plus acalabrutinib combination therapy, n=3 at baseline and n=2 at day 43. Green lines joining baseline and treatment paired samples reflect a >10% increase after treatment compared with baseline, red lines indicate a >10% decrease after treatment compared with baseline, and the black line (Panel B, right) indicates a change of <10% after treatment compared with baseline. ICOS, inducible co-stimulator; TiME, tumor-immune microenvironment.

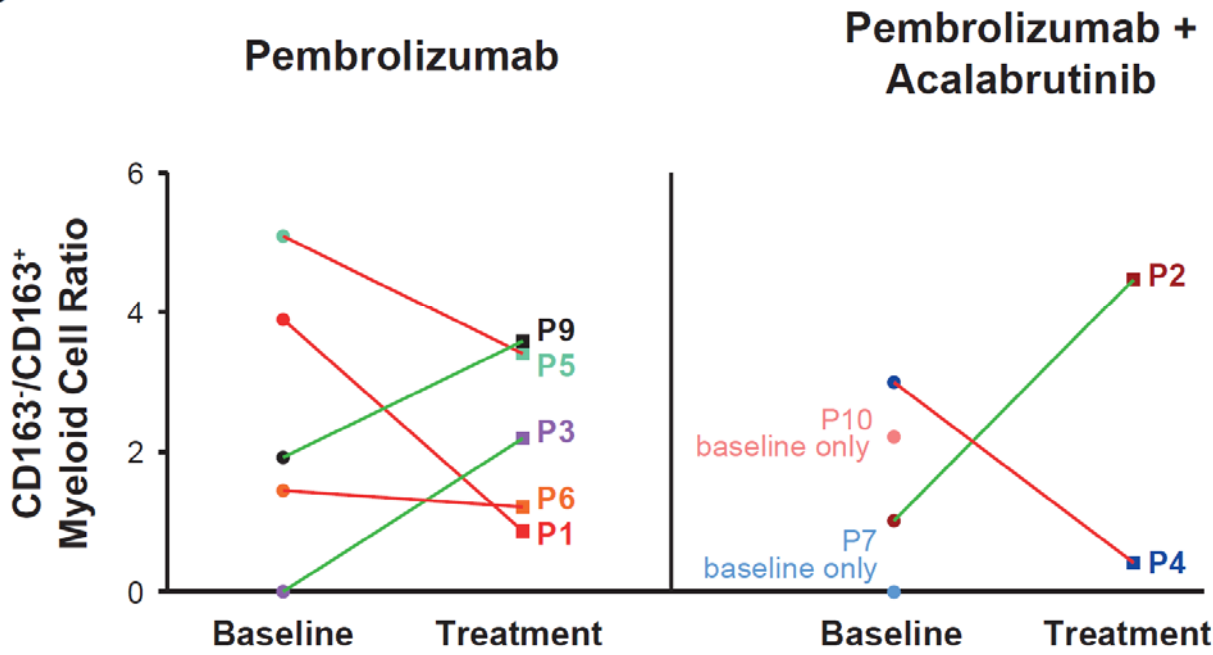
A



B



C



D

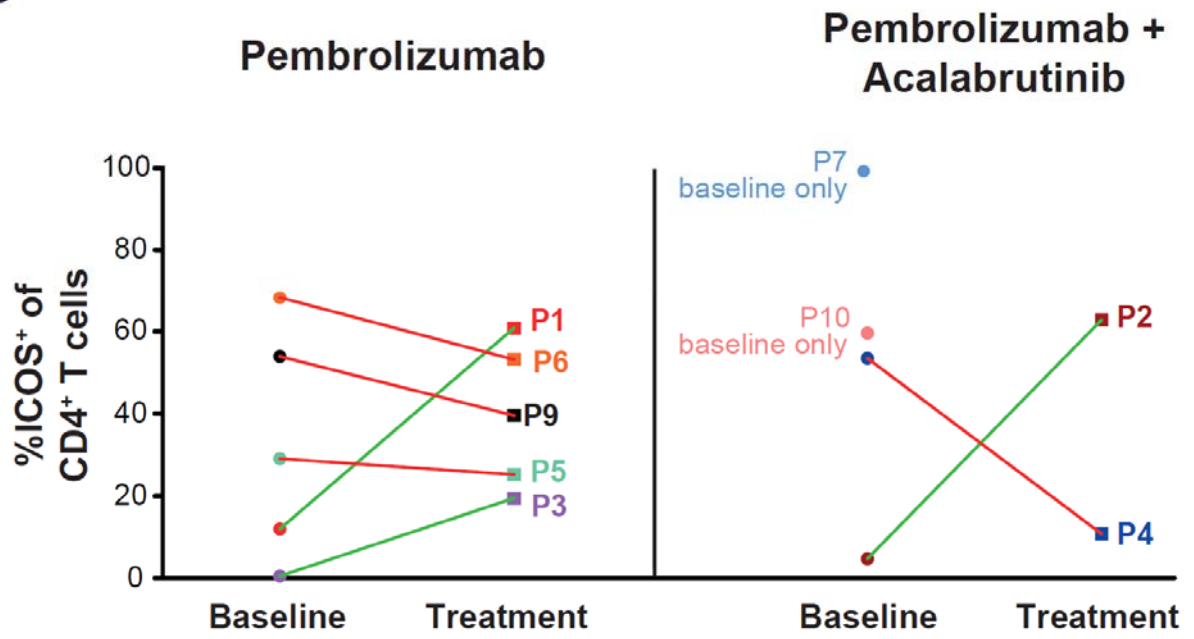
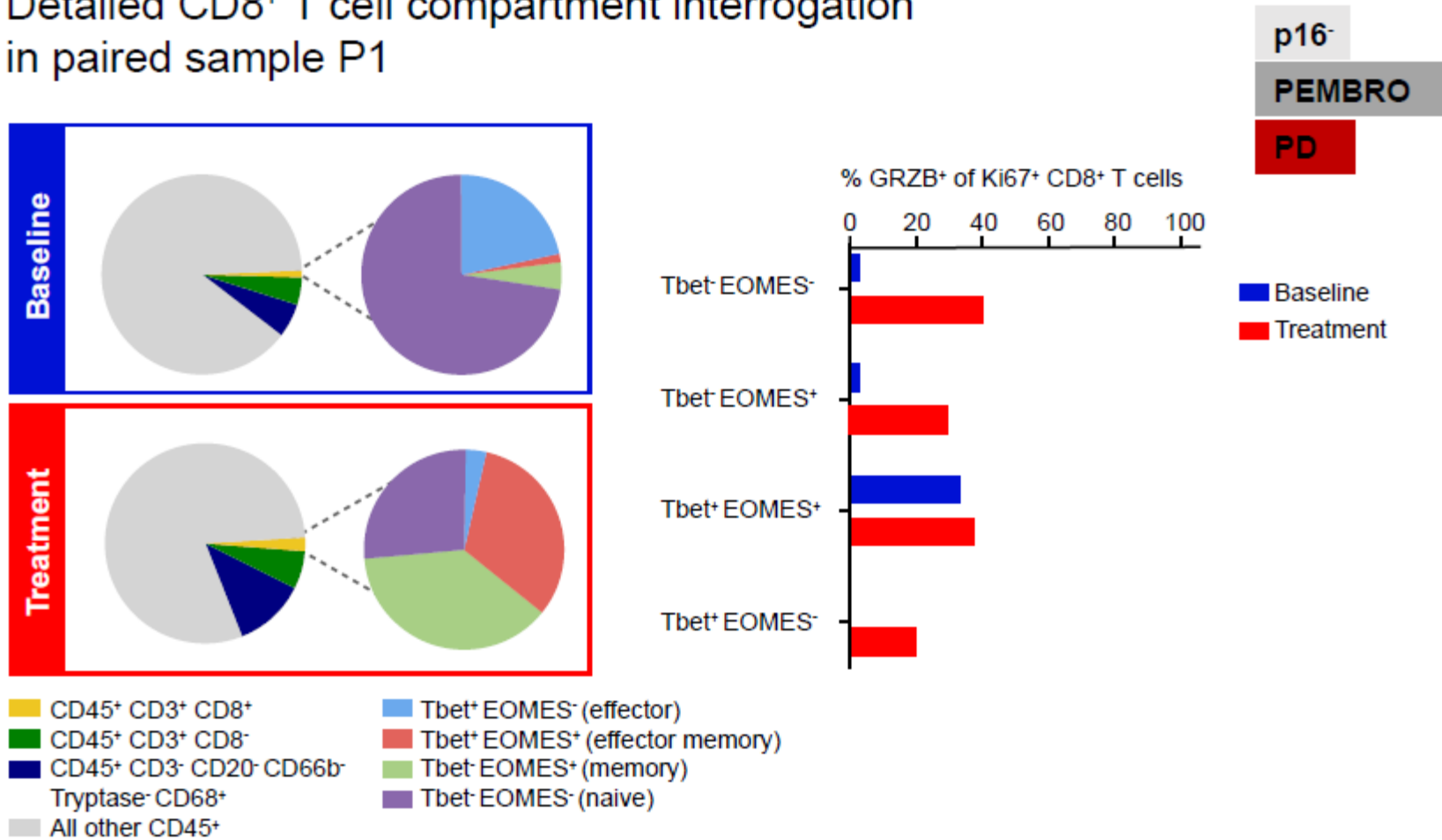
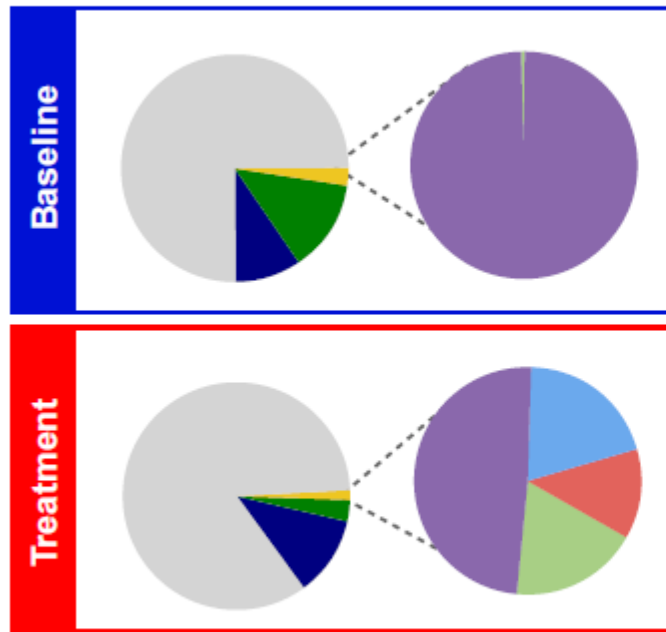


Figure S4. Detailed CD8⁺ T cell Compartment Interrogation.

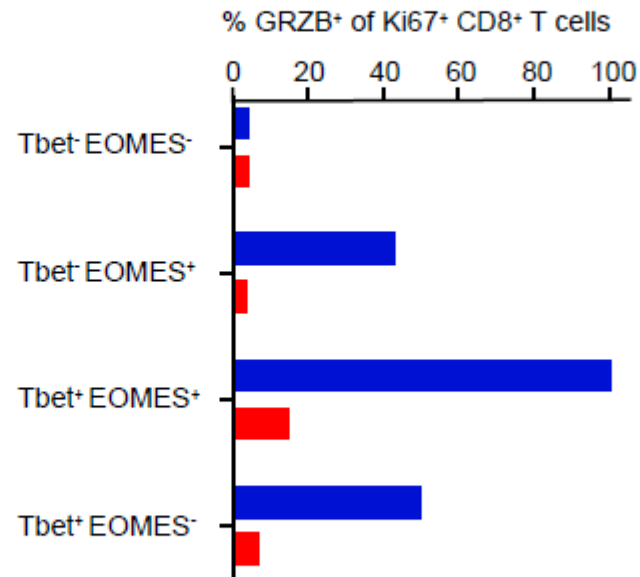
Detailed CD8⁺ T cell compartment interrogation in paired sample P1



Detailed CD8⁺ T cell compartment interrogation in paired sample P2



- CD45⁺ CD3⁺ CD8⁺
- CD45⁺ CD3⁺ CD8⁻
- CD45⁺ CD3⁻ CD20⁻ CD66b⁻ Tryptase⁻ CD68⁺
- All other CD45⁺
- Tbet⁺ EOMES⁻ (effector)
- Tbet⁺ EOMES⁺ (effector memory)
- Tbet⁻ EOMES⁺ (memory)
- Tbet⁻ EOMES⁻ (naive)



p16⁻

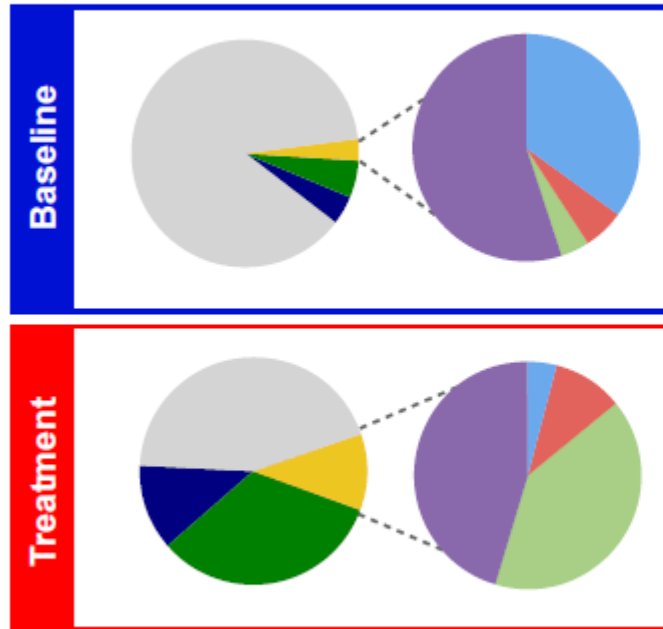
PEMBRO + Acalabrutinib

PD

■ Baseline

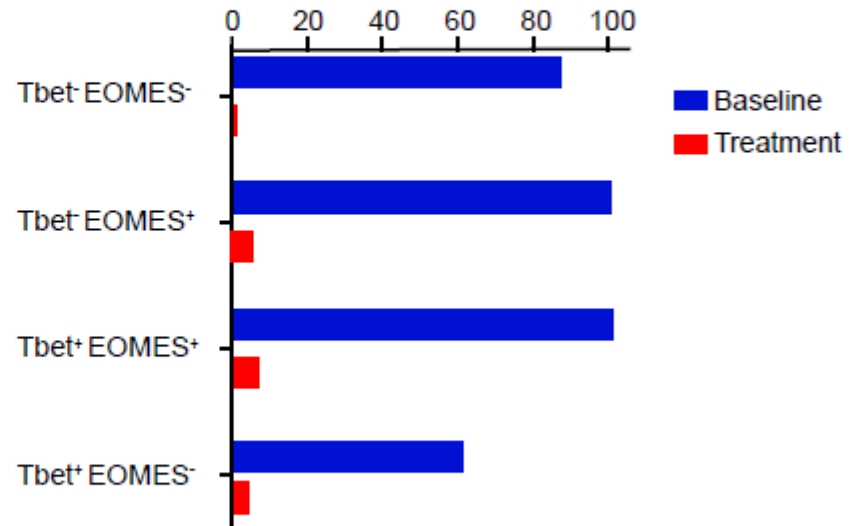
■ Treatment

Detailed CD8⁺ T cell compartment interrogation in paired sample P3



- CD8⁺ T cells
- Tbet⁺ EOMES⁻ (effector)
- CD4⁺ T cells
- Tbet⁺ EOMES⁺ (effector memory)
- CD68⁺ myelomonocytic
- Tbet⁻ EOMES⁺ (memory)
- Other CD45⁺ cells
- Tbet⁻ EOMES⁻ (naive)

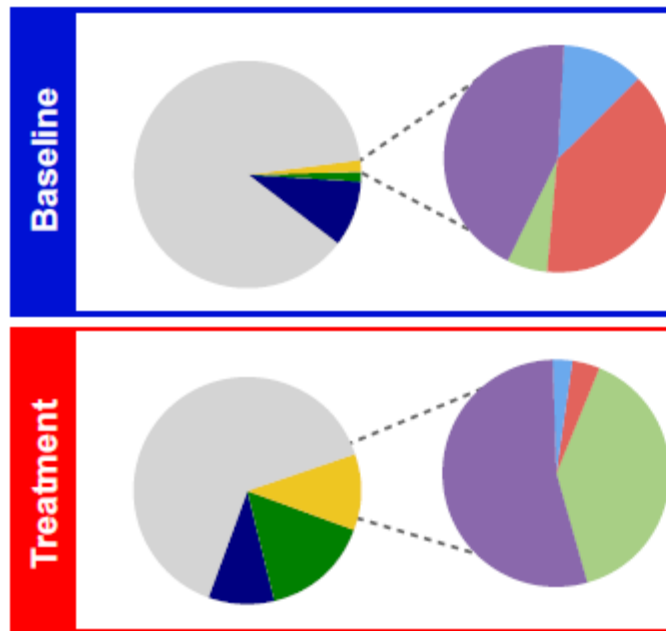
% GRZB⁺ of Ki67⁺ CD8⁺ T cells



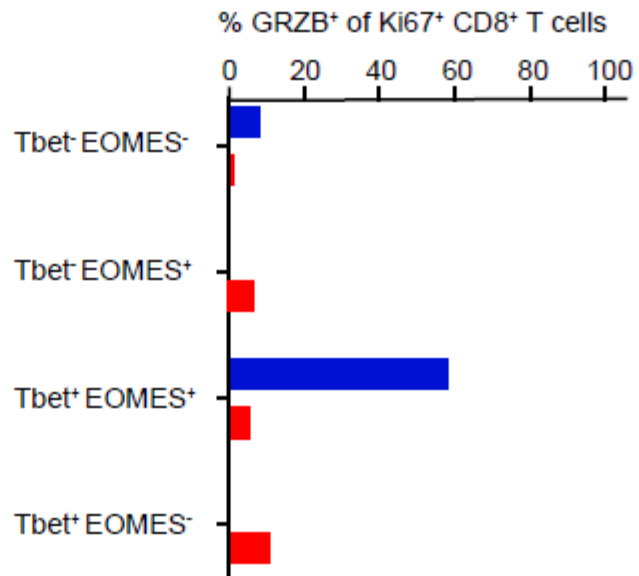
p16⁺
PEMBRO
PD

■ Baseline
■ Treatment

Detailed CD8⁺ T cell compartment interrogation in paired sample P4



- CD8⁺ T cells
- Tbet⁺ EOMES⁻ (effector)
- CD4⁺ T cells
- Tbet⁺ EOMES⁺ (effector memory)
- CD68⁺ myelomonocytic
- Tbet EOMES⁺ (memory)
- Other CD45⁺ cells
- Tbet EOMES⁻ (naive)



p16⁻

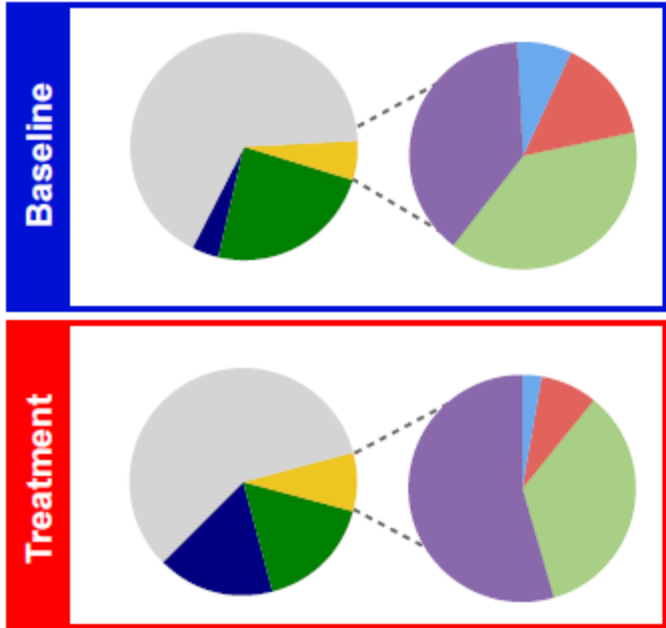
PEMBRO + Acalabrutinib

SD

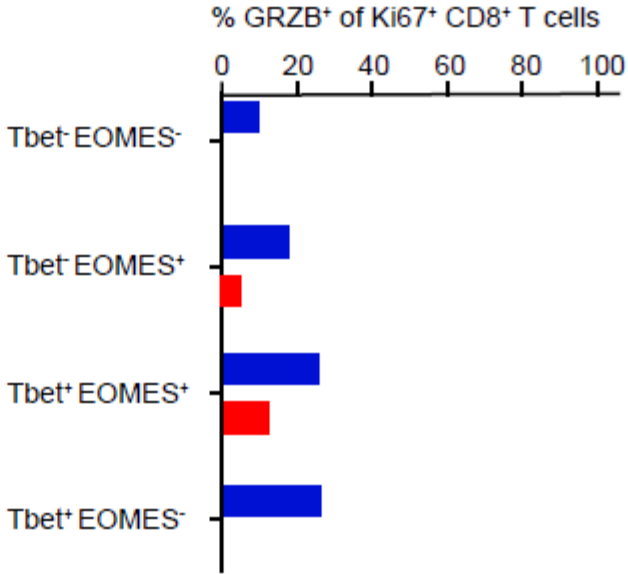
■ Baseline

■ Treatment

Detailed CD8⁺ T cell compartment interrogation in paired sample P5



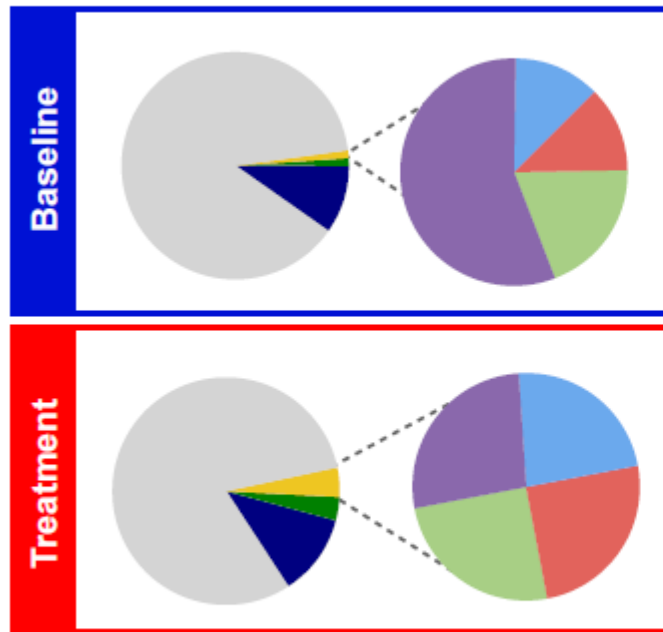
- CD45⁺ CD3⁺ CD8⁺
- CD45⁺ CD3⁺ CD8⁻
- CD45⁺ CD3⁻ CD20⁻ CD66b⁻ Tryptase⁻ CD68⁺
- All other CD45⁺
- Tbet⁺ EOMES⁻ (effector)
- Tbet⁺ EOMES⁺ (effector memory)
- Tbet⁻ EOMES⁺ (memory)
- Tbet⁻ EOMES⁻ (naive)



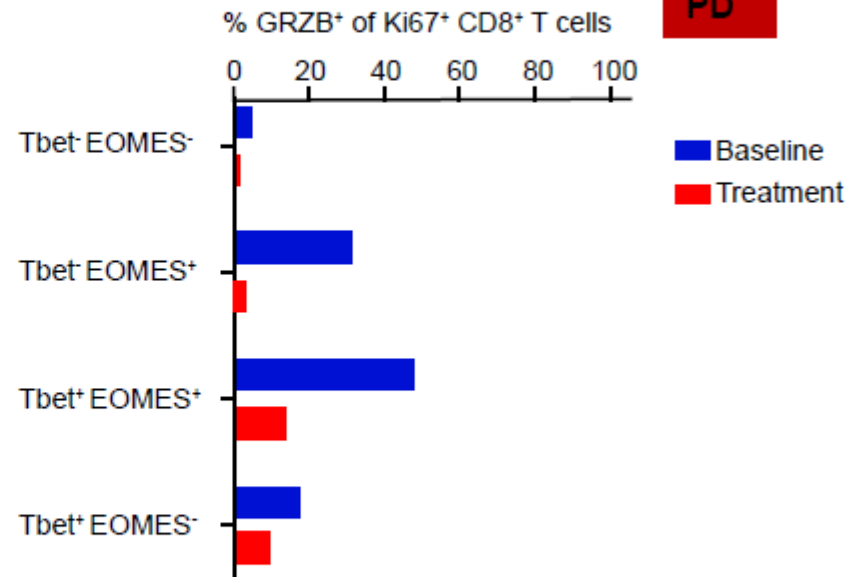
p16⁺
PEMBRO
PD

- Baseline
- Treatment

Detailed CD8⁺ T cell compartment interrogation in paired sample P6



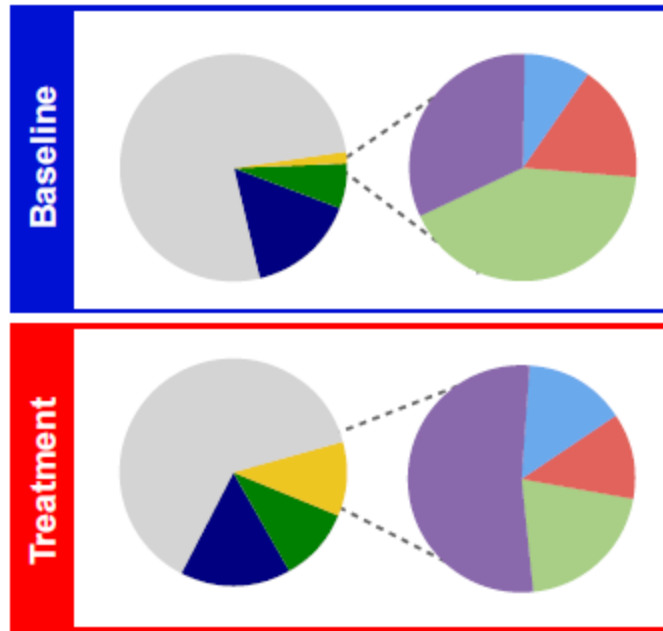
- CD45⁺ CD3⁺ CD8⁺
- CD45⁺ CD3⁺ CD8⁻
- CD45⁺ CD3⁻ CD20⁻ CD66b⁻
Tryptase⁻ CD68⁺
- All other CD45⁺
- Tbet⁺ EOMES⁻ (effector)
- Tbet⁺ EOMES⁺ (effector memory)
- Tbet⁻ EOMES⁺ (memory)
- Tbet⁻ EOMES⁻ (naive)



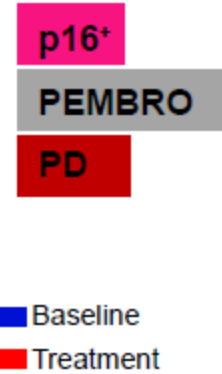
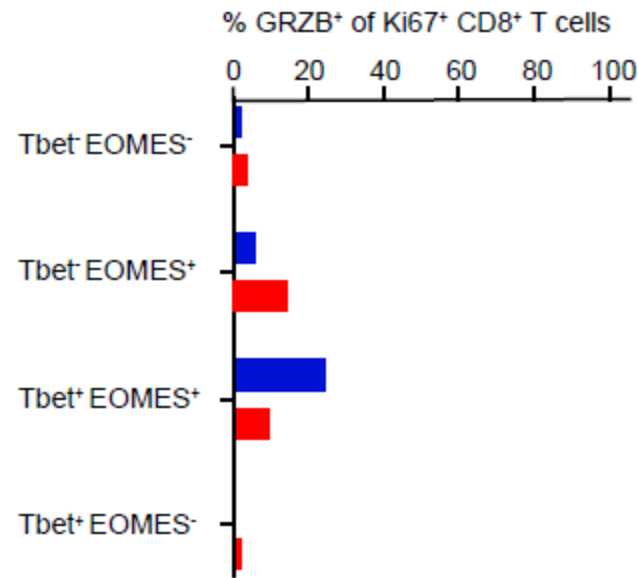
p16⁺
PEMBRO
PD

■ Baseline
■ Treatment

Detailed CD8⁺ T cell compartment interrogation in paired sample P9



- CD45⁺ CD3⁺ CD8⁺
- CD45⁺ CD3⁺ CD8⁻
- CD45⁺ CD3⁻ CD20⁻ CD66b⁻ Tryptase⁻ CD68⁺
- All other CD45⁺
- Tbet⁺ EOMES⁻ (effector)
- Tbet⁺ EOMES⁺ (effector memory)
- Tbet⁺ EOMES⁺ (memory)
- Tbet⁺ EOMES⁻ (naive)



Detailed CD8⁺ T cell compartment interrogation in paired sample P7 and P10 (baseline only)

