

# Supplementary Materials

## Methylation cytometric pretreatment blood immune profiles with tumor mutation burden as prognostic indicators for survival outcomes in head and neck cancer patients on anti-PD-1 therapy

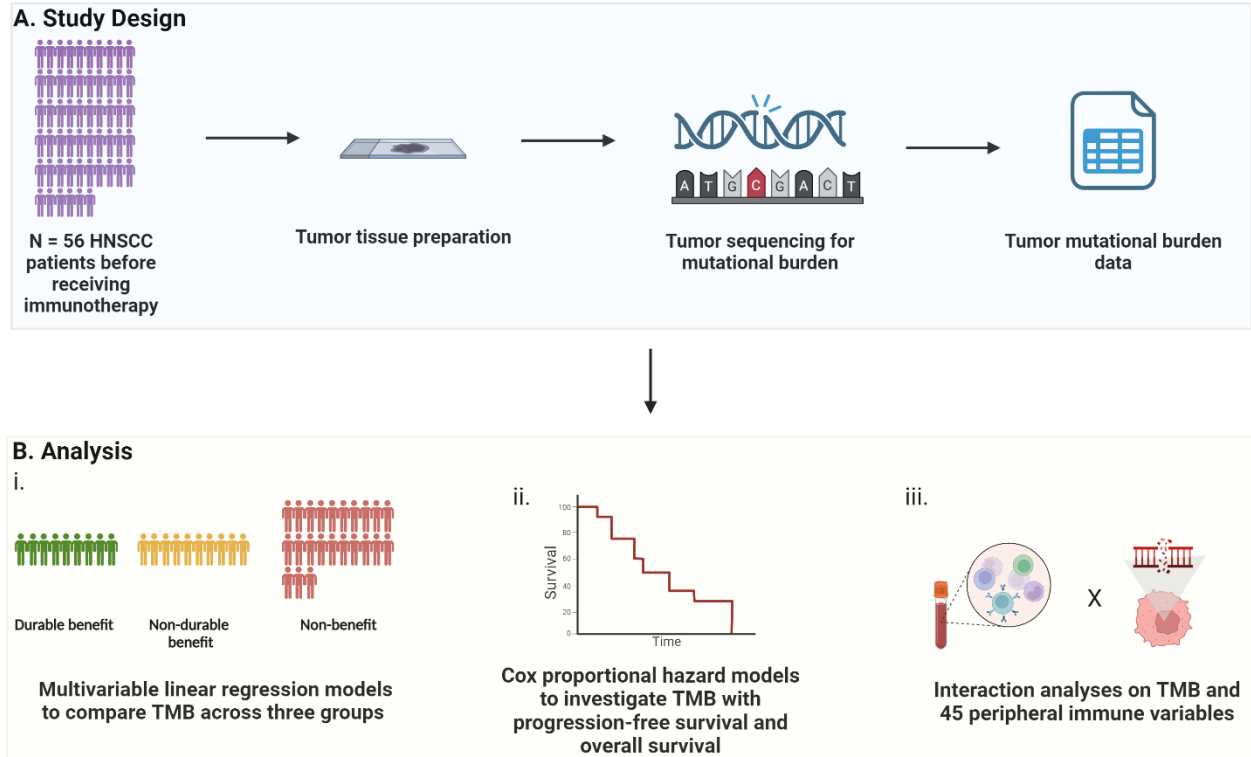
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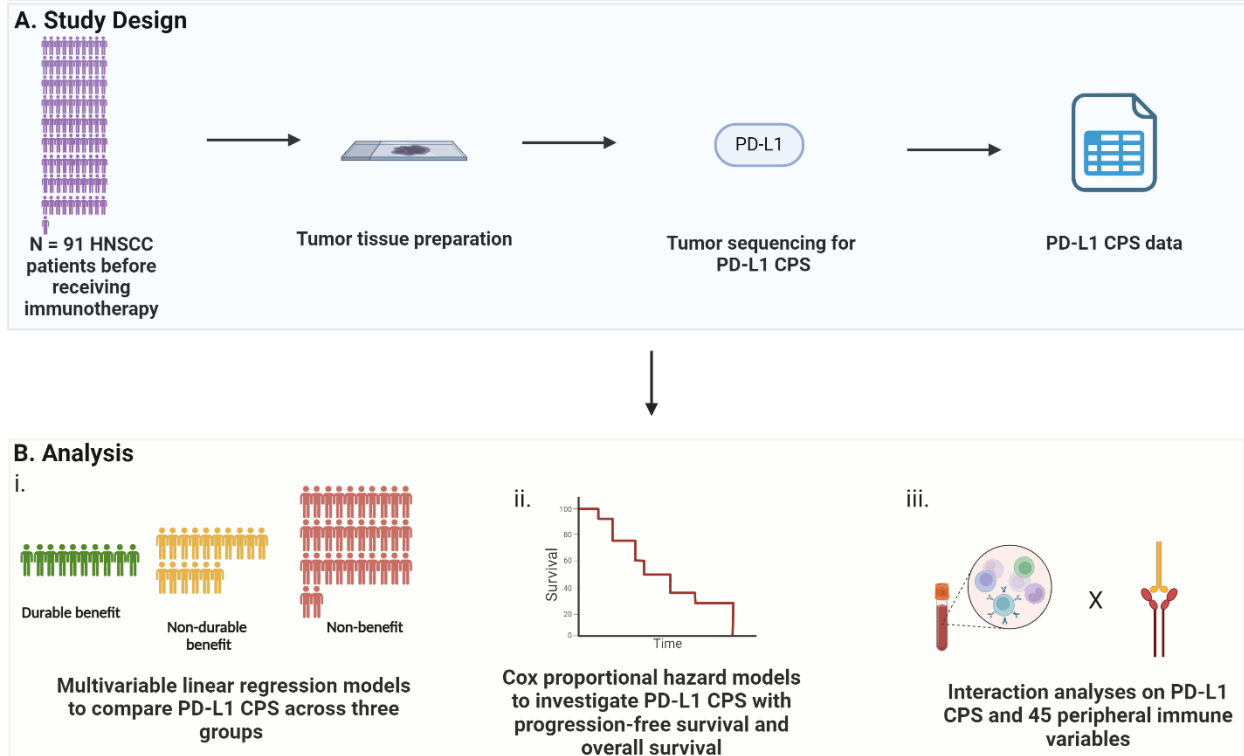
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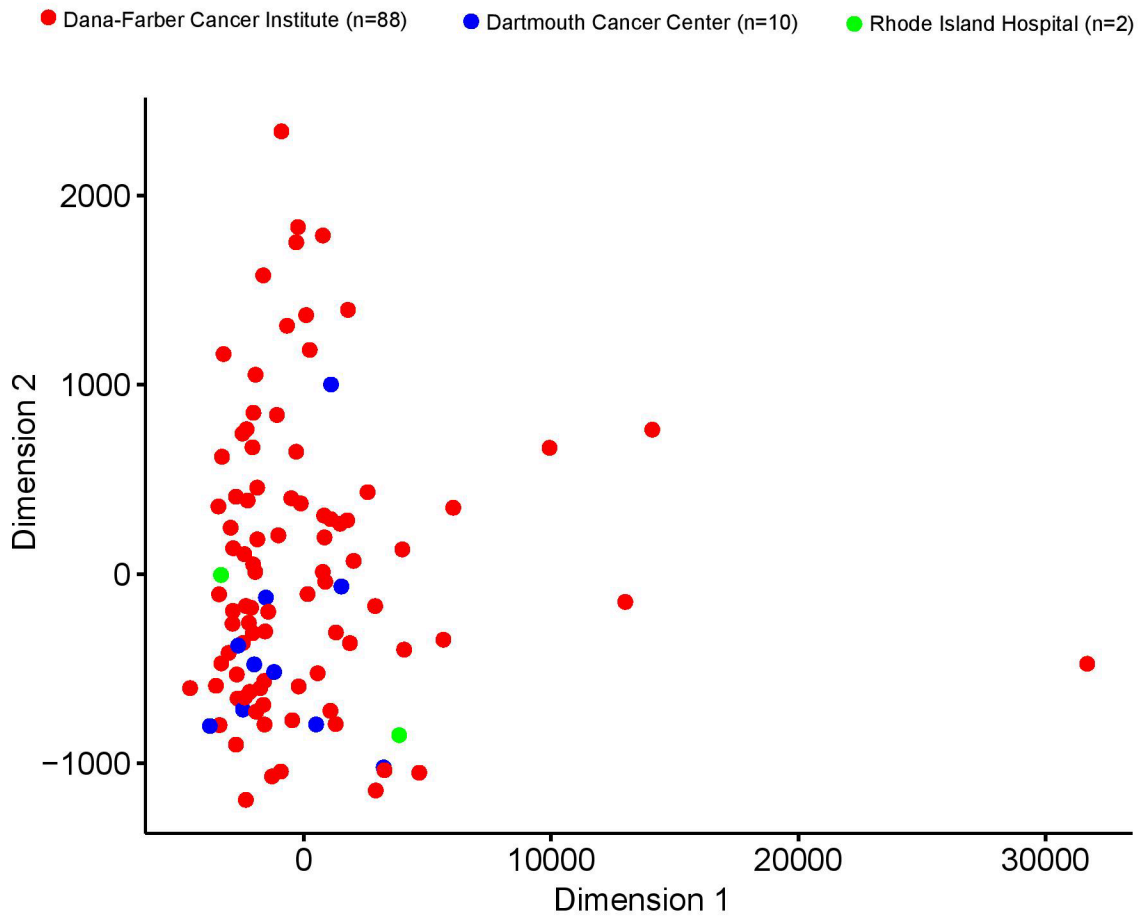
**Supplementary Table 6.** Significant TMB and peripheral immune variable interactions on **A.** progression-free survival; **B.** overall survival ..... 21



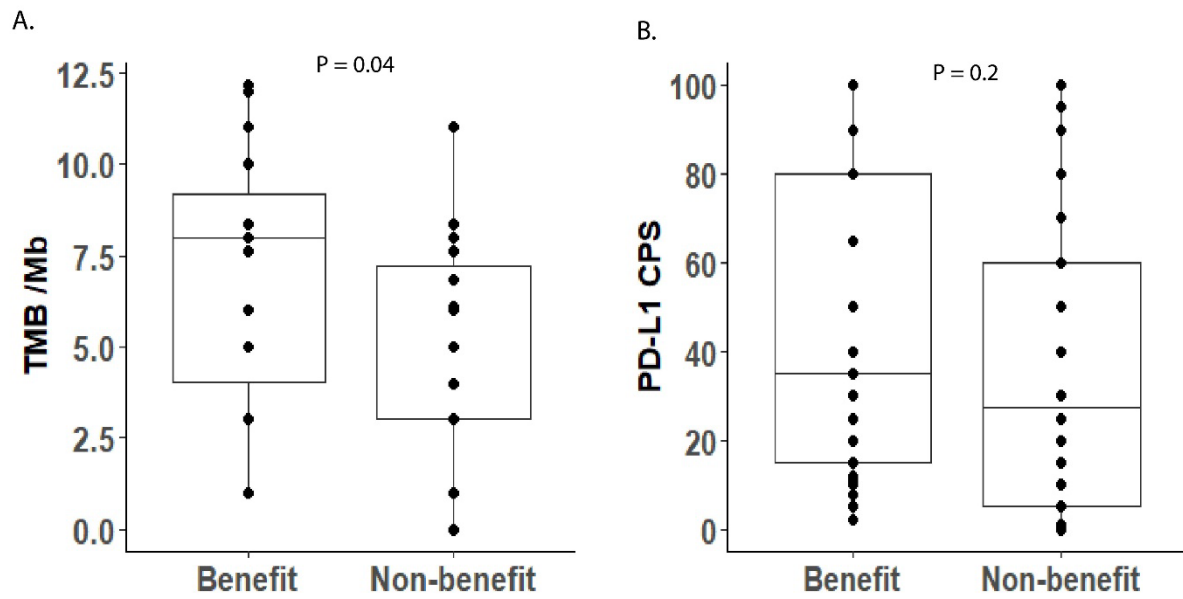
**Supplementary Figure 1.** Study design and analysis pipelines for TMB.



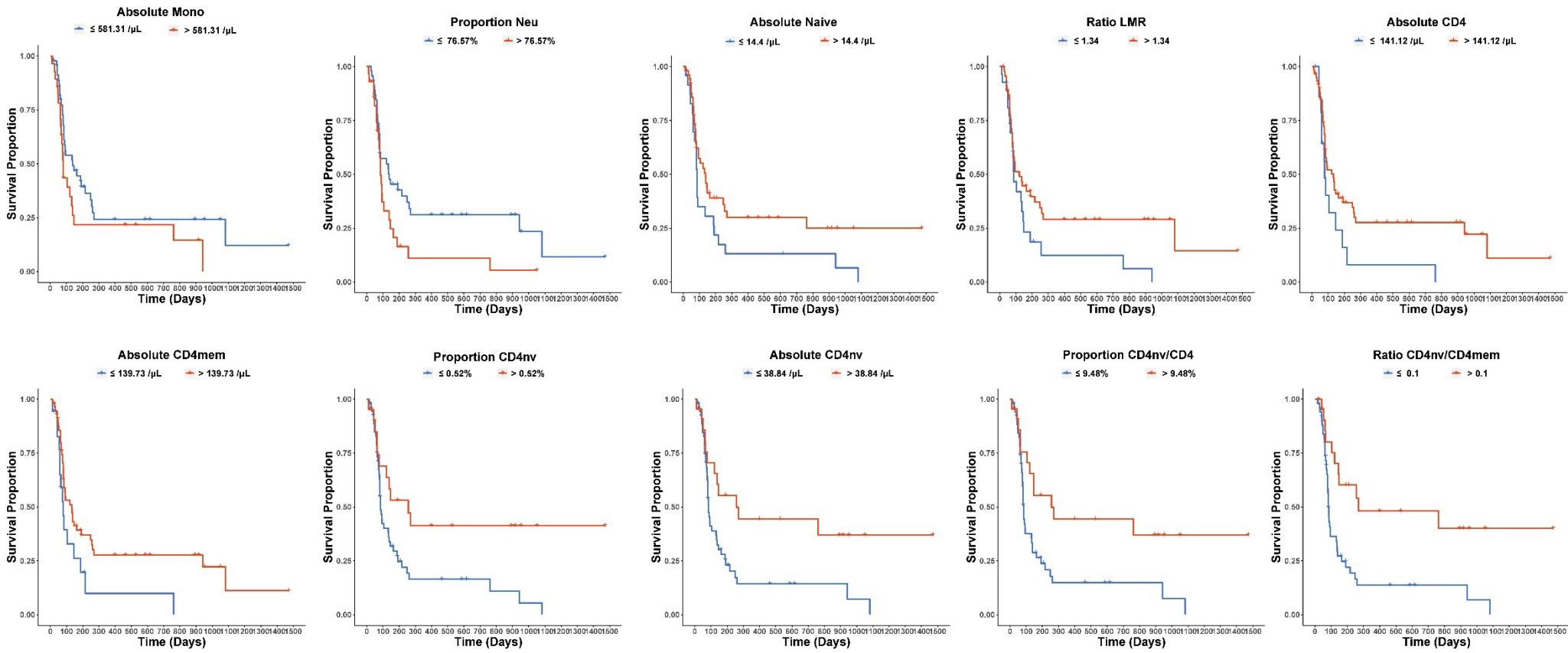
**Supplementary Figure 2.** Study design and analysis pipelines for PD-L1 CPS.



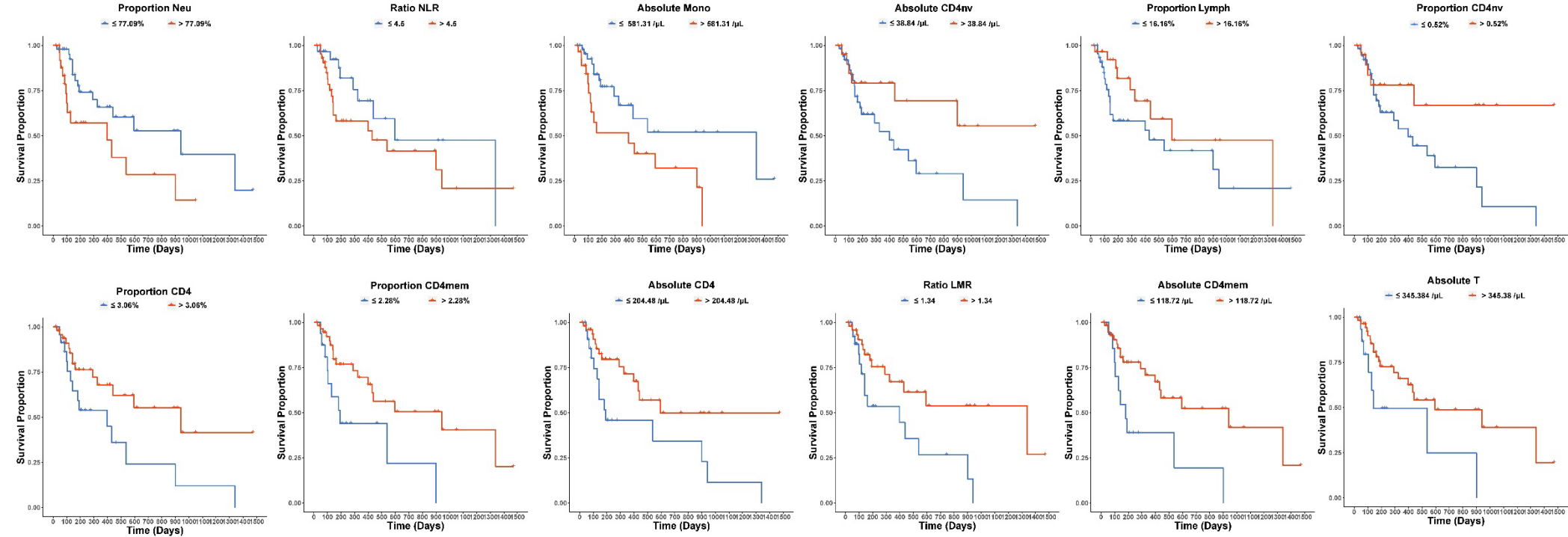
**Supplementary Figure 3.** Multidimensional scaling plot using baseline methylation cytometry immune profiles on all subjects colored by three centers.



**Supplementary Figure 4.** TMB and PDL1-CPS comparison between the benefit and non-benefit groups.

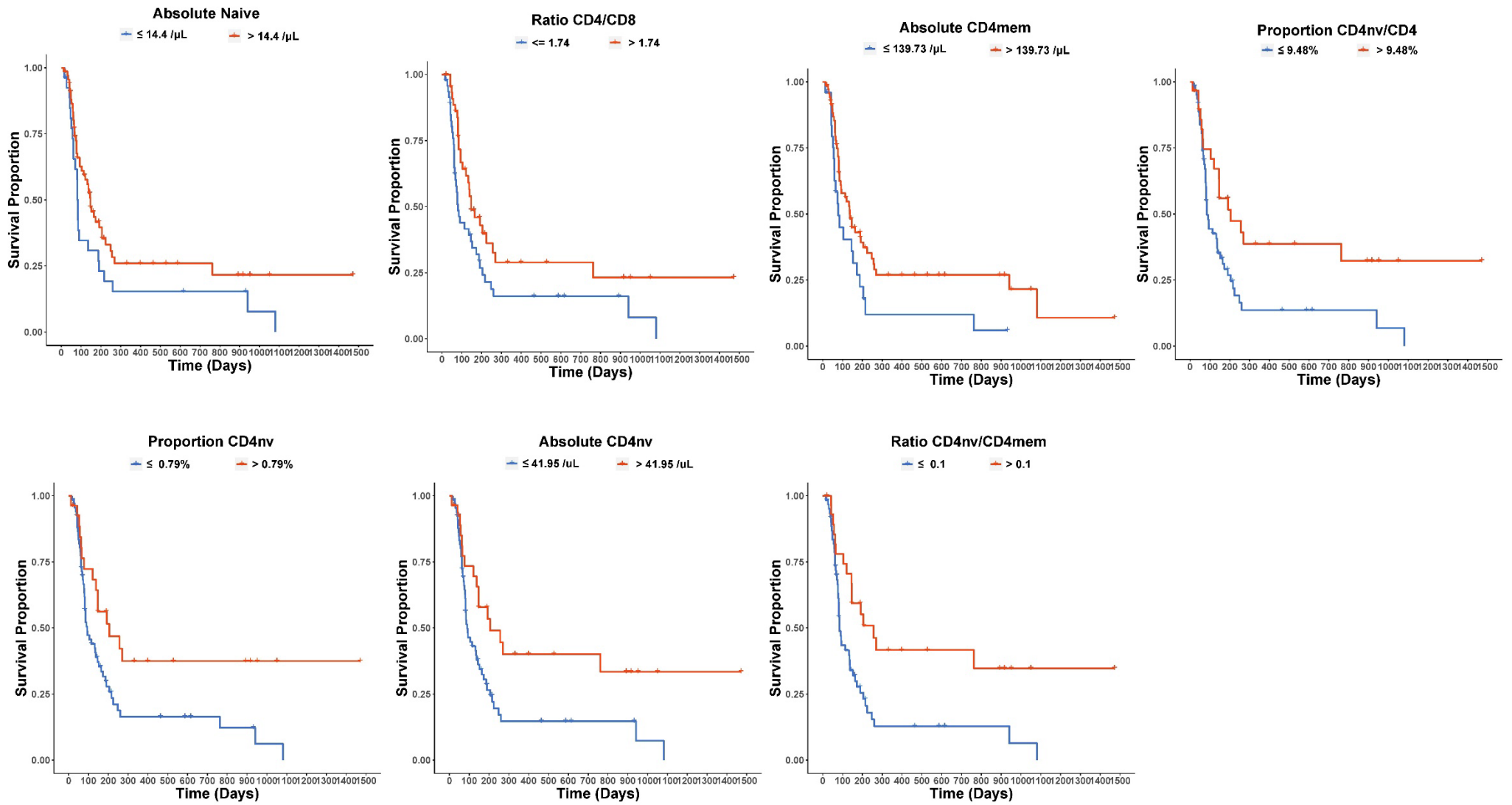


Supplementary Figure 5. Kaplan–Meier plots for progression-free survival in anti-PD-1 monotherapy.

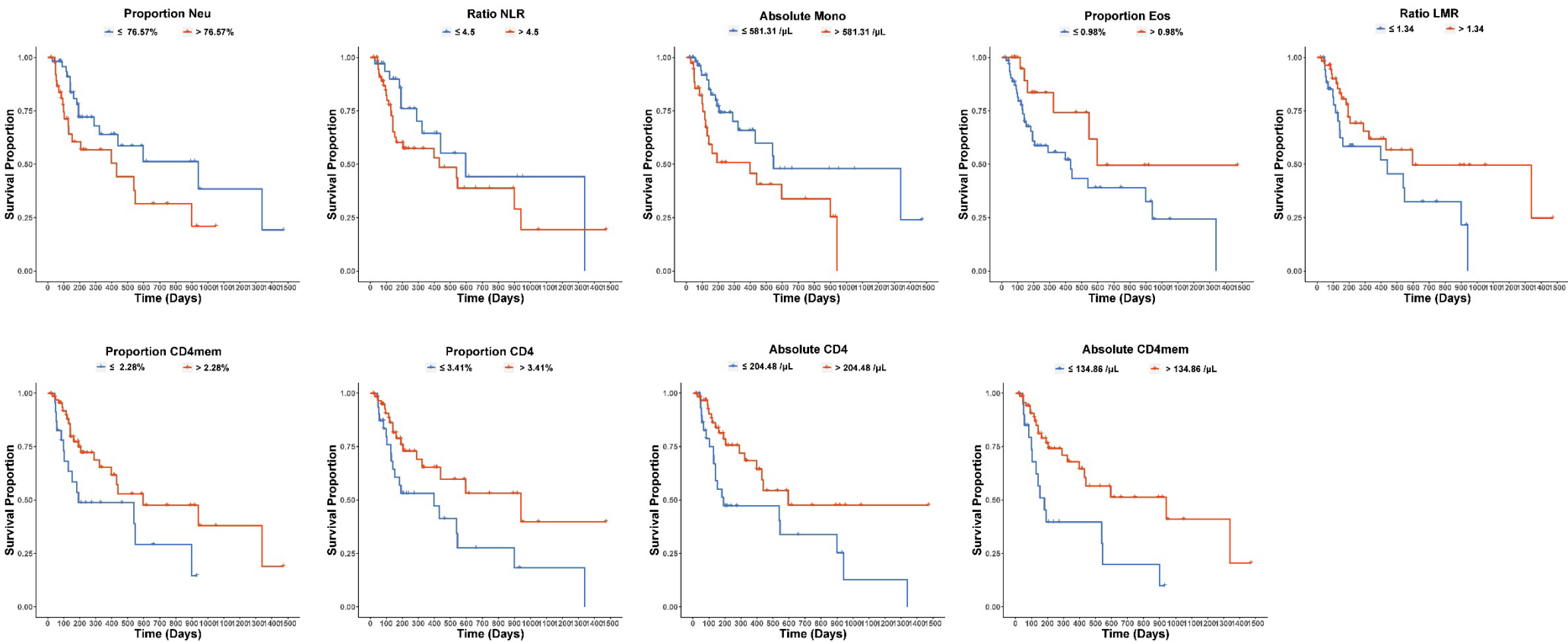


Supplementary Figure 6. Kaplan–Meier plots for overall survival in anti-PD-1 monotherapy.

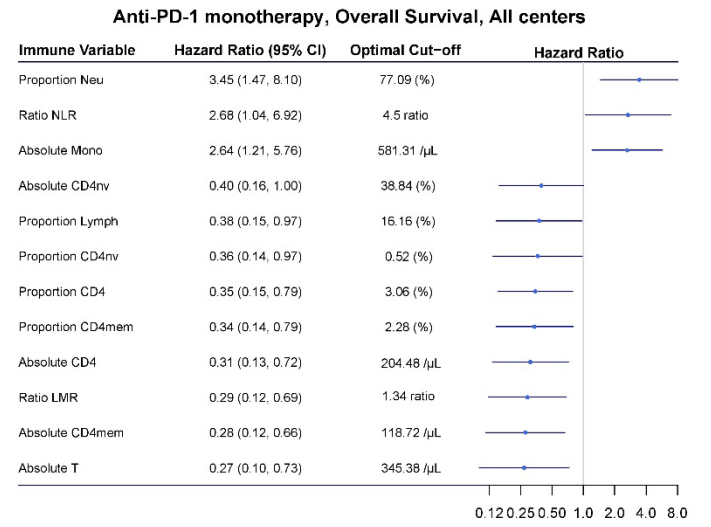
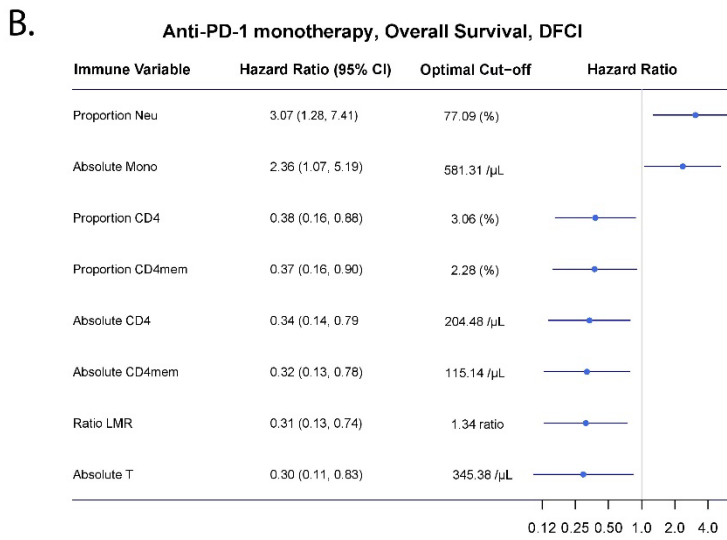
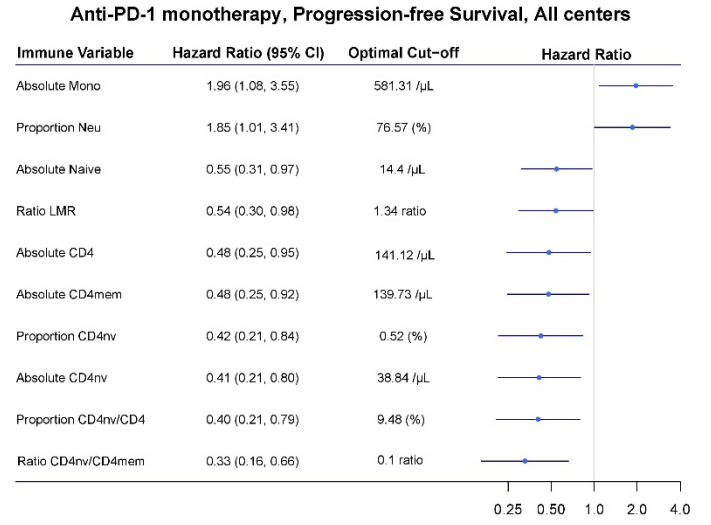
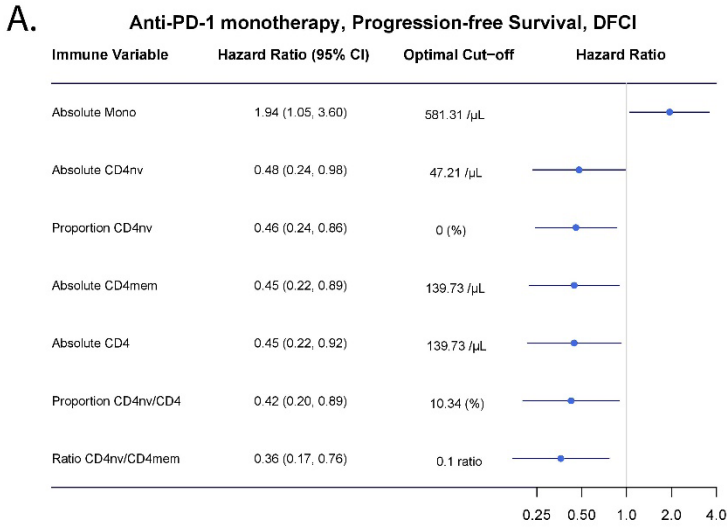




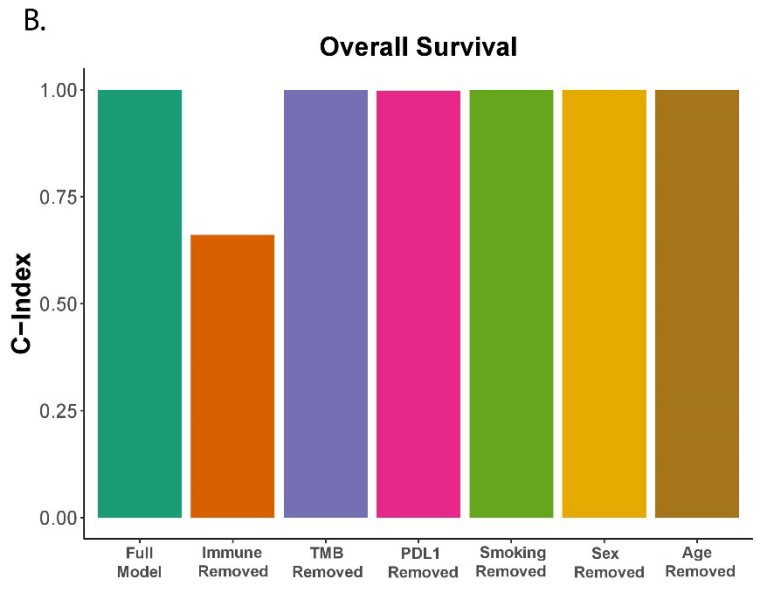
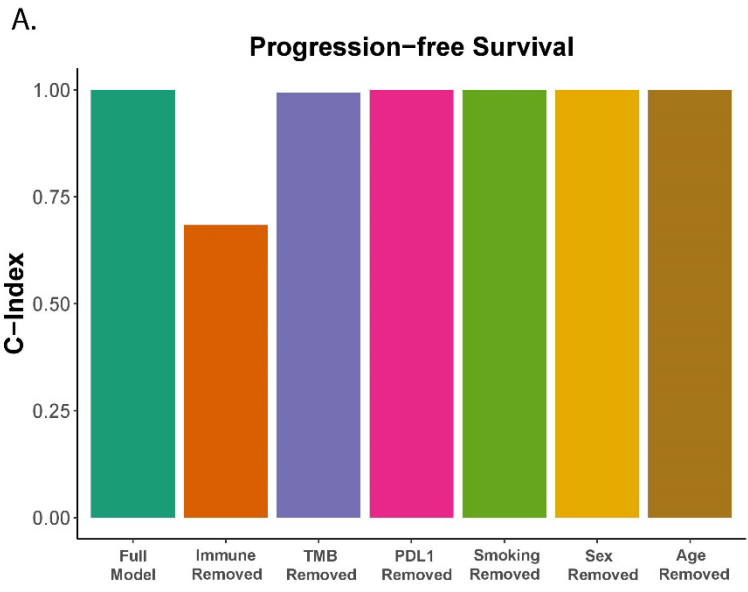
Supplementary Figure 7. Kaplan–Meier plots for progression-free survival in all-treatment.



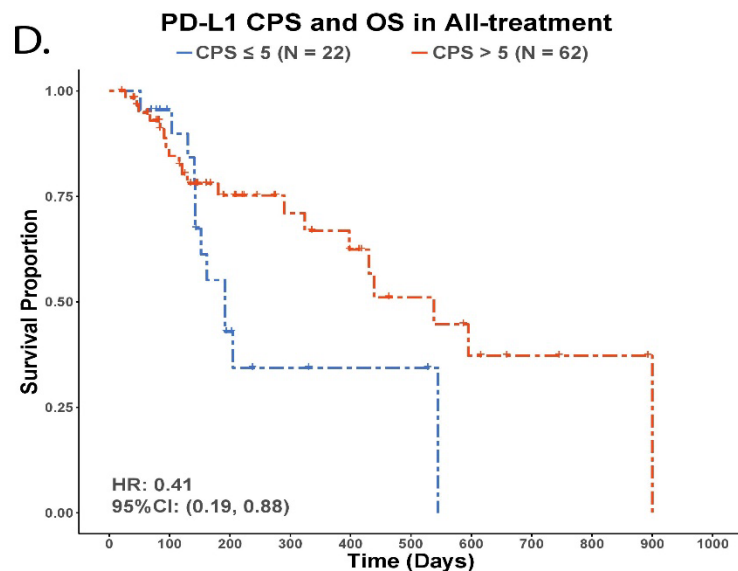
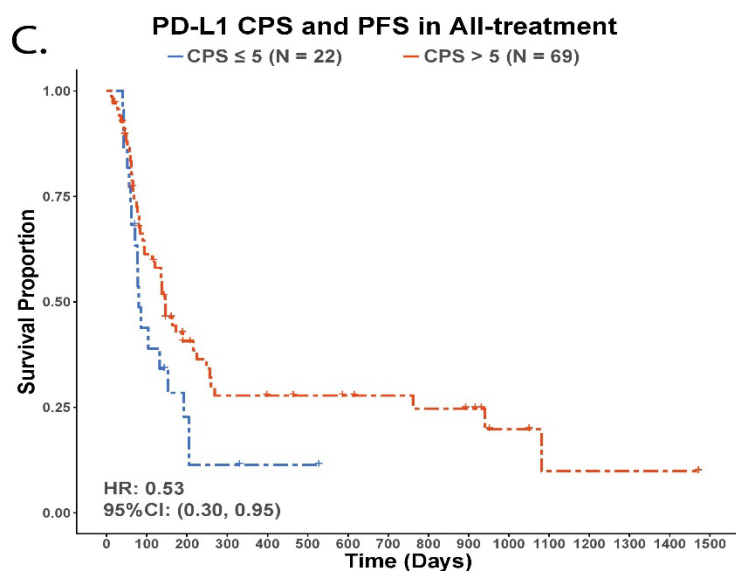
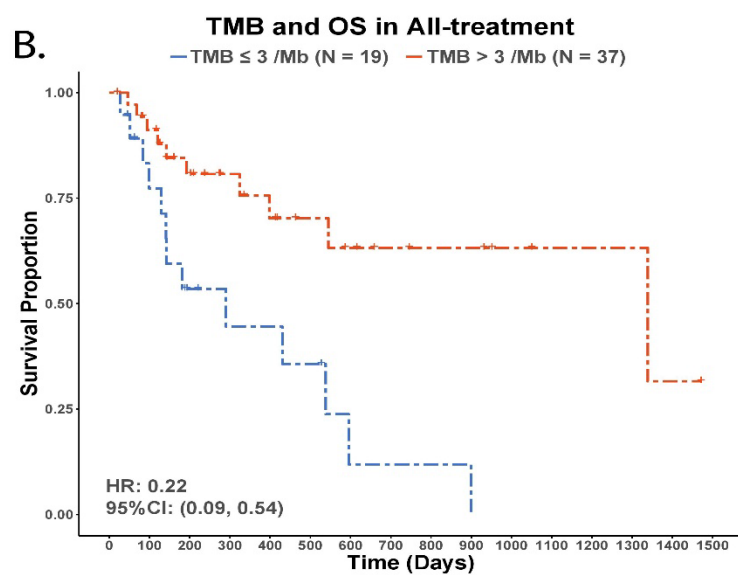
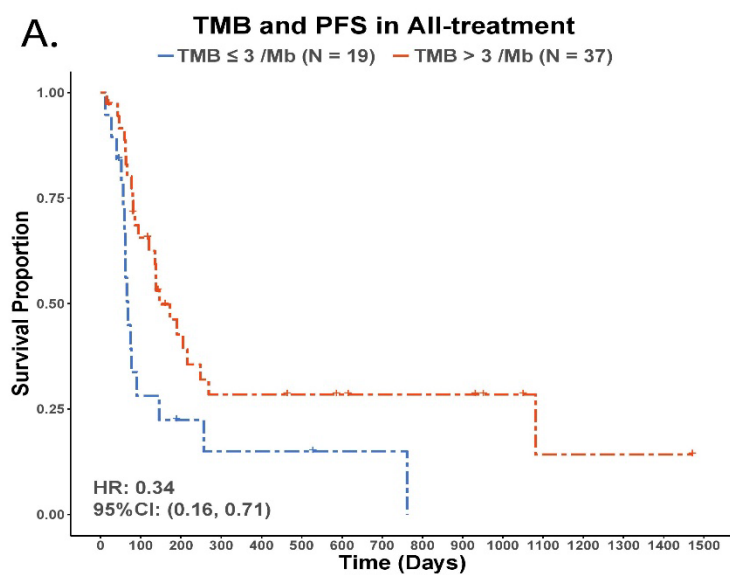
Supplementary Figure 8. Kaplan–Meier plots for overall survival in all-treatment.



**Supplementary Figure 9.** The association between immune variables and **A.** PFS and **B.** OS in DFCI patients vs all patients from three centers.

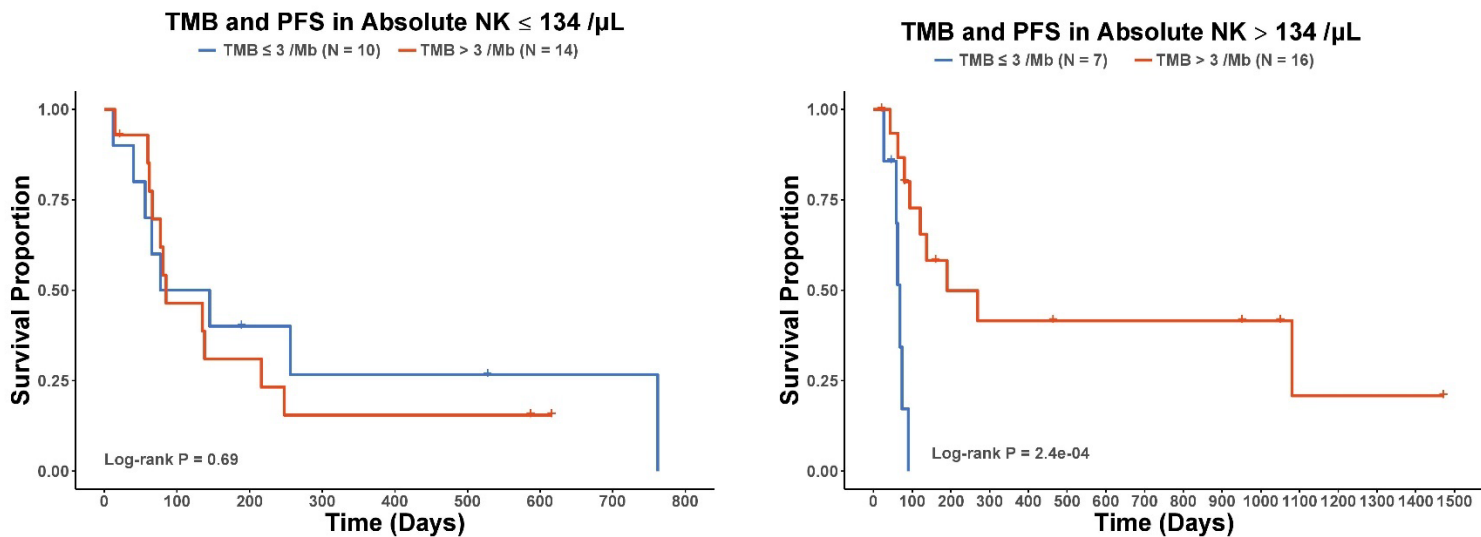


**Supplementary Figure 10.** C-index comparison between the full model and variable removed models on **A.** PFS; and **B.** OS.

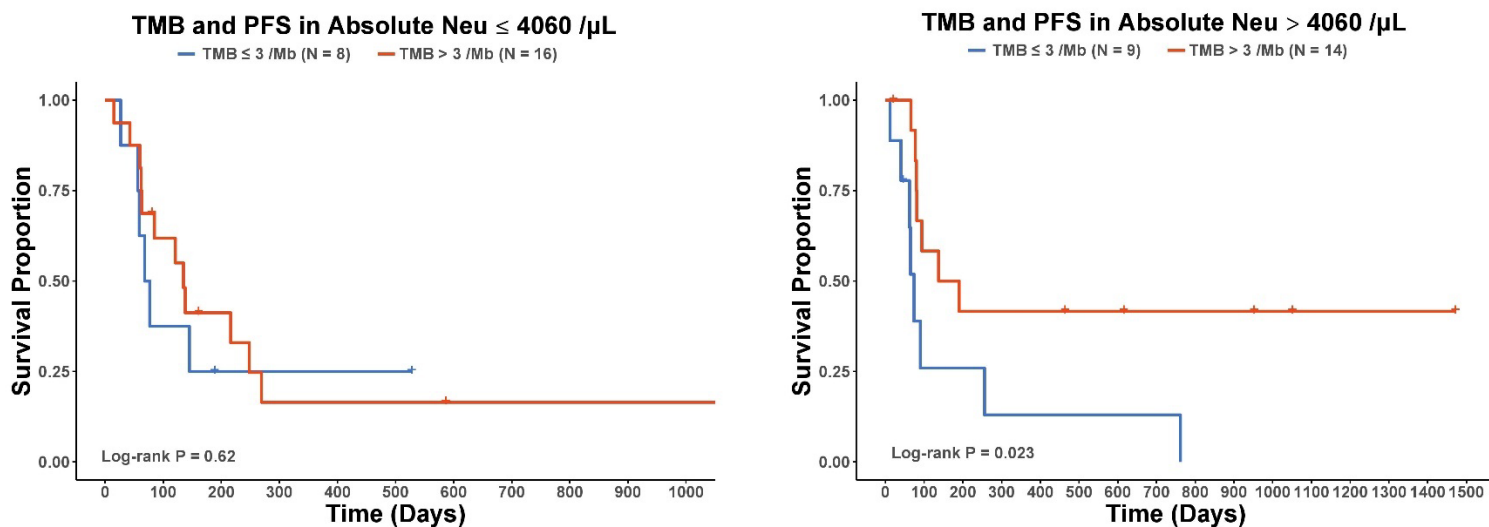


**Supplementary Figure 11.** TMB and PD-L1 CPS with survival outcomes in all-treatment.

### A. TMB and PFS in anti-PD-1 monotherapy stratified by median absolute NK count



### B. TMB and PFS in anti-PD-1 monotherapy stratified by median absolute Neu count

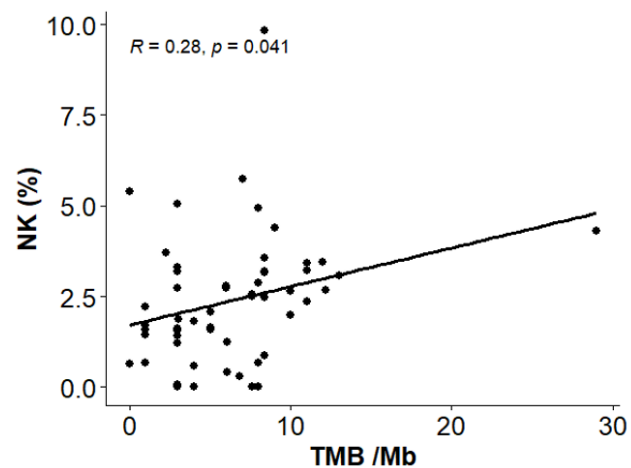


**Supplementary Figure 12.** TMB and progression-free survival stratified by median NK count and Neu count.

A.

Immune Variable	Pearson's r	P-value
Proportion NK	0.28	0.04
Proportion Mono	0.21	0.12
Ratio CD8nv/CD8mem	0.21	0.14
Absolute NK	0.20	0.15
Proportion CD4nv/CD4	-0.20	0.15

B.



**Supplementary Figure 13. A.** Top 5 association between pretreatment TMB and immune variables; **B.** Correlation scatter plot between pretreatment NK (%) and TMB with Pearson's correlation coefficient and p-value.

Type	Variable name	Description	Calculation
Proportion	Proportion Bas	Basophil proportion	proportion of basophils per extended deconvolution
Proportion	Proportion Bmem	B memory cell proportion	proportion of B memory cells per extended deconvolution
Proportion	Proportion Bnv	B naïve cell proportion	proportion of B naïve cells per extended deconvolution
Proportion	Proportion CD4mem	CD4T memory cell proportion	proportion of CD4T memory cells per extended deconvolution
Proportion	Proportion CD4nv	CD4T naïve cell proportion	proportion of CD4T naïve cells per extended deconvolution
Proportion	Proportion CD8mem	CD8T memory cell proportion	proportion of CD8memory cells per extended deconvolution
Proportion	Proportion CD8nv	CD8T naïve cell proportion	proportion of CD8naïve cells per extended deconvolution
Proportion	Proportion Eos	Eosinophil proportion	proportion of eosinophils per extended deconvolution
Proportion	Proportion Neu	Neutrophil proportion	proportion of neutrophils per extended deconvolution
Proportion	Proportion NK	Natural killer proportion	proportion of natural killer cells (NK cells) per extended deconvolution
Proportion	Proportion Mono	Monocyte proportion	proportion of monocytes per extended deconvolution
Proportion	Proportion Treg	T regulatory cell proportion	proportion of Tregs per extended deconvolution
Derived Proportion	Proportion CD4nv/CD4	CD4T naïve over total CD4T cell proportion	CD4 naïve/Total CD4 (naïve + memory + Treg) -- calculated using proportions * 100
Derived Proportion	Proportion CD8nv/CD8	CD8T naïve over total CD8T cell proportion	CD8naïve/Total CD8 (naïve + memory) -- calculated using proportions * 100
Derived Proportion	Proportion Treg/CD4	Treg over total CD4T cell proportion	Treg/Total CD4 (naïve + memory + Treg) -- calculated using proportions * 100
Derived Proportion	Proportion Bnv/B	B naïve over total B cell proportion	B naïve/total B cell (naïve+memory B cells) -- calculated using proportions * 100
Derived Proportion	Proportion B	Total B cell proportion	Bnv + Bmem -- calculated using proportions
Derived Proportion	Proportion CD4	Total CD4T cell proportion	CD4nv + CD4mem + Treg -- calculated using proportions
Derived Proportion	Proportion CD8	Total CD8T cell proportion	CD8nv + CD8mem -- calculated using proportions
Derived Proportion	Proportion Naive	Total naïve cell proportion	Bnv + CD4nv + CD8nv -- calculated using proportions
Derived Proportion	Proportion Lymph	Total lymphocyte proportion	NK + Bmem + Bnv + CD4mem + CD4nv + CD8mem + CD8nv +Treg -- calculated using proportions
Derived Proportion	Proportion Naive/Lymph	Total naïve cell over lymphocyte proportion	totNaive/totLymph -- calculated using proportions * 100
Absolute Count	Absolute Bas	Absolute basophil count	Absolute basophil count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute Bmem	Absolute B memory cell count	Absolute B memory count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute Bnv	Absolute B naïve cell count	Absolute B naïve count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute CD4mem	Absolute CD4T memory cell count	Absolute CD4T memory count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute CD4nv	Absolute CD4T naïve cell count	Absolute CD4T naïve count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute CD8mem	Absolute CD8T memory cell count	Absolute CD8T memory count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute CD8nv	Absolute CD8T naïve cell count	Absolute CD8T naïve count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute Eos	Absolute eosinophil count	Absolute eosinophil count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute Mono	Absolute monocyte count	Absolute monocyte count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute Neu	Absolute neutrophil count	Absolute neutrophil count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute NK	Absolute natural killer cell count	Absolute natural killer cell (NK) count per ul --calculated by multiplying proportion with the total cell count
Absolute Count	Absolute Treg	Absolute T regulatory cell count	Absolute Treg count per ul --calculated by multiplying proportion with the total cell count
Derived Count	Absolute Lymph	Absolute lymphocyte cell count	Counts of NK + B naïve + B memory + CD4 naïve +CD4 memory+ CD8 naïve + CD8 memory + Treg -- calculated using absolute counts
Derived Count	Absolute T	Absolute total T cell count	Counts of CD4 naïve+CD4 memory + CD8 naïve+CD8 memory + Treg -- calculated using absolute counts
Derived Count	Absolute CD4	Absolute total CD4T cell count	(CD4nv + CD4mem + Treg)*Total_Cell_Count --calculated by multiplying proportion with the total cell count
Derived Count	Absolute CD8	Absolute total CD8T cell count	(CD8nv + CD8mem)*Total_Cell_Count --calculated by multiplying proportion with the total cell count
Derived Count	Absolute B	Absolute total B cell count	(Bnv + Bmem) * Total_Cell_Count --calculated by multiplying proportion with the total cell count
Derived Count	Absolute Naive	Absolute total naïve cell count	(Bnv + CD4nv + CD8nv)* Total_Cell_Count --calculated by multiplying proportion with the total cell count
Derived Ratio	Ratio CD4/CD8	CD4T to CD8T cell ratio	Total CD4 (naïve + memory +Treg)/total CD8 (naïve + memory) -- calculated using proportions
Derived Ratio	Ratio CD4nv/CD4mem	CD4T naïve to memory cell ratio	CD4 naïve/CD4 memory -- calculated using proportions
Derived Ratio	Ratio CD8nv/CD8mem	CD8T naïve to memory cell ratio	CD8naïve/CD8 memory -- calculated using proportions
Derived Ratio	Ratio NLR	Neutrophil to lymphocyte ratio	Neutrophil/ (NK +B naïve + B memory +CD4 naïve + CD4 memory +CD8 naïve + CD8 memory +Treg) -- calculated using proportions
Derived Ratio	Ratio LMR	Lymphocyte to monocyte ratio	(NK+B naïve + B memory + CD4 naïve +CD4 memory+ CD8 naïve + CD8 memory +Treg) / monocyte --calculated using proportions

**Supplementary Table 1.** Immune variables abbreviations, and their calculations.



	Mean		Median		Percentile range (2.5–97.5)	
	WHI	HNSCC	WHI	HNSCC	WHI	HNSCC
<b>WBC</b>	6276	7207	6000	5850	(3387, 10680)	(3200, 17808)
<b>Neutrophil</b>						
Cells/ $\mu$ l	3642	5595	3467	4256	(1334, 7202)	(2387, 15635)
%	56.8	75.1	57.5	75.7	(31.6, 76.8)	(59.2, 91.8)
<b>Basophil</b>						
Cells/ $\mu$ l	66	27	42	0	(18, 243)	(0, 254)
%	1.1	0.36	0.6	0	(0.48, 3.5)	(0, 2.4)
<b>Eosinophil</b>						
Cells/ $\mu$ l	140	74	112	7	(19, 444)	(0, 594)
%	2.3	1.1	2	0.1	(0.42, 6.5)	(0, 7.6)
<b>Monocyte</b>						
Cells/ $\mu$ l	546	605	524	548	(262, 961)	(272, 1192)
%	8.9	9.3	8.7	8.7	(4.9, 14.3)	(3.7, 16.6)
<b>Lymphocyte</b>						
Cells/ $\mu$ l	1921	906	1823	878	(842, 3621)	(194, 1910)
%	31.5	14.2	30.1	13.5	(14.4, 54.1)	(2.9, 27.0)
<b>CD4T</b>						
Cells/ $\mu$ l	860	376	795	318	(296, 1799)	(2, 974)
%	14.1	5.9	13.3	5.1	(2.8, 21.2)	(0, 15.5)
<b>CD4T naïve</b>						
Cells/ $\mu$ l	200	46	132	6	(35, 686)	(0, 251)
%	3.3	0.7	2.3	0.1	(0.9, 11.4)	(0, 3.9)
<b>CD4T memory</b>						
Cells/ $\mu$ l	610	323	564	248	(171, 1328)	(0, 787)
%	10.1	5.1	9.4	5	(2.8, 21.2)	(0, 11.9)
<b>T regulatory</b>						
Cells/ $\mu$ l	50	6.5	47	0	(27, 88)	(0, 87)
%	0.8	0.1	0.78	0	(0.78, 0.78)	(0, 0.9)
<b>CD8T</b>						
Cells/ $\mu$ l	499	275	397	171	(85, 1473)	(0.4, 1011)
%	8.1	4.3	6.7	3.5	(1.6, 22.4)	(0, 14)
<b>CD8T naïve</b>						
Cells/ $\mu$ l	80	6	58	0	(28, 247)	(0, 73)
%	1.3	0.1	0.8	0	(0.8, 4.1)	(0, 1)
<b>CD8T memory</b>						
Cells/ $\mu$ l	418	269	306	163	(37, 1386)	(0.4, 1011)
%	6.8	4.2	5.3	3.2	(0.8, 21.6)	(0, 14)
<b>B cell</b>						
Cells/ $\mu$ l	227	87	189	53	(50, 640)	(0, 408)
%	3.8	1.3	3.2	0.9	(0.81, 9.8)	(0, 4.2)
<b>B naïve</b>						
Cells/ $\mu$ l	140	61	107	21	(23, 503)	(0, 368)
%	2.3	0.91	1.8	0.34	(0.48, 7.1)	(0, 3.8)
<b>B memory</b>						
Cells/ $\mu$ l	87	26	67	14	(16, 256)	(0, 129)
%	1.4	0.41	1.2	0.22	(0.32, 4.1)	(0, 1.9)
<b>Natural killer</b>						
Cells/ $\mu$ l	335	169	308	148	(95, 762)	(0, 422)
%	5.5	2.7	5.1	2.5	(1.6, 11)	(0, 6.8)

**Supplementary Table 2.** Immune profile comparison between HNSCC patients and WHI subjects.

<b>TMB Subjects' Characteristics</b>	<b>N=56</b>	<b>%</b>
<b>Recruitment Site</b>		
Dana-Farber Cancer Institute	56	100.0
Dartmouth Cancer Center	0	0
Rhode Island Hospital	0	0
<b>Sex</b>		
Female	12	21.4
Male	44	78.6
<b>Age, in years</b>		
Mean (standard deviation)	64.1 (12.4)	
<b>Location of primary tumor</b>		
Oral cavity	28	50.0
Oropharynx	19	33.9
Larynx	4	7.1
Hypopharynx	1	1.8
Paranasal sinuses and nasal cavity	2	3.6
Unknown	2	3.6
<b>HPV status in oropharyngeal carcinomas (=19)</b>		
Positive	18	94.7
Negative	1	5.3
Missing	0	0
<b>Smoking History</b>		
Current/former smoker	27	48.2
Never smoker	25	44.6
Missing	4	7.1
<b>Initial Treatment Type</b>		
Immunotherapy only	49	87.5
Immunotherapy + Chemotherapy	6	10.7
Immunotherapy + EGFRi	1	1.8
<b>Progression-free survival, in days</b>		
Median	135	
<b>Overall survival, in days</b>		
Median	545	
<b>Vital Status</b>		
Alive	33	58.9
Deceased	23	41.1
<b>Still continuing treatment among alive (=33)</b>		
Yes	26	78.8
No	7	21.2

**Supplementary Table 3.** Baseline characteristics of the study population for TMB.

<b>PDL1 Subjects' Characteristics</b>	<b>N=91</b>	<b>%</b>
<b>Recruitment Site</b>		
Dana-Farber Cancer Institute	85	93.4
Dartmouth Cancer Center	4	4.4
Rhode Island Hospital	2	2.2
<b>Sex</b>		
Female	24	26.4
Male	67	73.6
<b>Age, in years</b>		
Mean (standard deviation)	64.5 (11.8)	
<b>Location of primary tumor</b>		
Oral cavity	39	42.9
Oropharynx	31	34.1
Larynx	7	7.7
Hypopharynx	7	7.7
Paranasal sinuses and nasal cavity	3	3.3
Unknown	4	4.4
<b>HPV status in oropharyngeal carcinomas (=31)</b>		
Positive	24	77.4
Negative	6	19.4
Missing	1	3.2
<b>Smoking History</b>		
Current/former smoker	48	52.7
Never smoker	38	41.8
Missing	5	5.5
<b>Initial Treatment Type</b>		
Immunotherapy only	73	80.2
Immunotherapy + Chemotherapy	17	18.7
Immunotherapy + EGFRi	1	1.1
<b>Progression-free survival, in days</b>		
Median	135	
<b>Overall survival, in days</b>		
Median	545	
<b>Vital Status</b>		
Alive	57	62.6
Deceased	34	37.4
<b>Still continuing treatment among alive (=57)</b>		
Yes	43	75.4
No	14	24.6

**Supplementary Table 4.** Baseline characteristics of the study population for PD-L1 CPS.

A.

Immune Variable	HR	Lower	Higher	P-value	Cut-off	FDR
Ratio CD4nv/CD4mem	0.3278	0.1629	0.6596	0.0018	0.1	0.0684
Proportion CD4nv/CD4	0.4046	0.2065	0.7924	0.0083	9.48	0.114
Absolute CD4nv	0.4118	0.2115	0.8017	0.009	38.84	0.114
Proportion CD4nv	0.4232	0.2142	0.836	0.0133	0.52	0.12635
Absolute Mono	1.9609	1.0842	3.5466	0.0259	581.31	0.170729
Absolute CD4mem	0.4788	0.2484	0.923	0.0279	139.73	0.170729
Absolute CD4	0.4829	0.2457	0.949	0.0347	141.12	0.170729
Absolute Naive	0.5459	0.3086	0.9657	0.0375	14.4	0.170729
Ratio LMR	0.5393	0.2969	0.9797	0.0426	1.34	0.170729
Proportion Neu	1.8505	1.0054	3.4061	0.048	76.57	0.170729

B.

Immune Variable	HR	Lower	Higher	P-value	Cut-off	FDR
Absolute CD4mem	0.2782	0.1167	0.663	0.0039	118.72	0.05985
Proportion Neu	3.4455	1.4661	8.0977	0.0045	77.09	0.05985
Ratio LMR	0.2909	0.1234	0.6856	0.0048	1.34	0.05985
Absolute CD4	0.3102	0.1339	0.7187	0.0063	204.48	0.05985
Absolute T	0.2708	0.1004	0.7304	0.0099	345.38	0.068875
Proportion CD4	0.3456	0.1516	0.7883	0.0116	3.06	0.068875
Proportion CD4mem	0.3389	0.1446	0.7945	0.0128	2.28	0.068875
Absolute Mono	2.641	1.2123	5.7535	0.0145	581.31	0.068875
Ratio NLR	2.6837	1.0412	6.917	0.041	4.5	0.153036
Proportion Lymph	0.3767	0.1458	0.9737	0.0439	16.16	0.153036
Proportion CD4nv	0.3641	0.136	0.9747	0.0443	0.52	0.153036
Absolute CD4nv	0.3953	0.1565	0.9985	0.0496	38.84	0.157067

C.

Immune Variable	HR	Lower	Higher	P-value	Cut-off	FDR
Ratio CD4nv/CD4mem	0.4245	0.2346	0.768	0.0046	0.1	0.1444
Absolute CD4nv	0.4726	0.2634	0.848	0.012	41.95	0.1444
Proportion CD4nv/CD4	0.4888	0.2749	0.869	0.0148	9.48	0.1444
Proportion CD4nv	0.4827	0.2655	0.8774	0.0169	0.79	0.1444
Ratio CD4/CD8	0.5347	0.317	0.902	0.019	1.74	0.1444
Absolute CD4mem	0.5119	0.2831	0.9257	0.0267	139.73	0.1691
Absolute Naive	0.5868	0.3479	0.9898	0.0457	14.4	0.183667

D.

Immune Variable	HR	Lower	Higher	P-value	Cut-off	FDR
Absolute CD4mem	0.2837	0.1319	0.6105	0.0013	134.86	0.0494
Absolute CD4	0.3113	0.1432	0.677	0.0032	204.48	0.0608
Proportion Neu	2.9858	1.3702	6.5067	0.0059	76.57	0.074733
Proportion CD4	0.3673	0.1736	0.7772	0.0088	3.41	0.0836
Proportion CD4mem	0.4016	0.1902	0.8477	0.0167	2.28	0.12692
Ratio LMR	0.4101	0.1927	0.8729	0.0207	1.34	0.1311
Absolute Mono	2.1683	1.1056	4.2526	0.0243	581.31	0.131914
Ratio NLR	2.3629	1.0126	5.5139	0.0467	4.5	0.199289
Proportion Eos	0.4088	0.169	0.9888	0.0472	0.98	0.199289

**Supplementary Table 5.** Significant associations between immune variables as binary variables and **A.** progression-free survival in anti-PD-1 monotherapy; **B.** overall survival in anti-PD-1 monotherapy; **C.** progression-free survival in all-treatment; **D.** overall survival in all-treatment.

A.

<b>Immune Variable</b>	<b>HR</b>	<b>Lower</b>	<b>Higher</b>	<b>P-value</b>	<b>FDR</b>
Absolute NK	0.9982	0.9968	0.9997	0.0154	0.53775
Absolute Neu	1	0.9999	1	0.0239	0.53775

B.

<b>Immune Variable</b>	<b>HR</b>	<b>Lower</b>	<b>Higher</b>	<b>P-value</b>	<b>FDR</b>
Proportion Mono	1.0657	1.0227	1.1104	0.0025	0.1125
Proportion CD4	1.0485	1.006	1.0928	0.0248	0.511364
Absolute NK	0.998	0.996	0.9999	0.0425	0.511364

**Supplementary Table 6.** Significant TMB and peripheral immune variable interactions on **A.** progression-free survival; **B.** overall survival.