Supplementary material

CD8$^+$PD-1$^+$T cells and PD-L-1$^+$ CTCs in chemotherapy-naïve NSCLC: Towards their clinical relevance?

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Supplementary Table 1.   Phenotypes and percentages of effector immune cells subpopulations in NSCLC patients according to histology

Cell types	Phenotype	% Mean value ± SEM		
		Non-SQ	SQ	P value
CD4+ T	CD3+CD4+	47.01 ± 2.92	52.32 ± 4.02	0.28
	CD3+CD4+PD-1+	39.29 ± 2.69	42.30 ± 3.64	0.50
	CD3+CD4+PD-L1+	1.96 ± 0.17	2.564 ± 0.26	**0.02**
CD8+ T	CD3+CD8+	27.86 ± 2.1	24.79 ± 3.47	0.42
	CD3+CD8+PD-1+	17.17 ± 1.8	16.04 ± 2.9	0.73
	CD3+CD8+PD-L1+	0.87 ± 0.11	0.60 ± 0.13	0.09
B cells	CD3-CD19+	22.47 ± 3.19	31.36 ± 6,24	0.1693
	CD3-CD19+PD-1+	1,11 ± 0,26	0.8846 ± 0,25	0.6425
	CD3-CD19+PD-L1+	0,28 ± 0,12	0,21 ± 0,06	0.5769
DC/ monocytes	CD14+HLA-DR+CD3-CD56-CD19-	83.54 ± 3.47	88.01 ± 3.19	0.11
	CD14+HLA-DR+CD3-CD56-CD19-PD-1+	19.96 ± 2.22	15.40 ± 2.98	0.12
	CD14+HLA-DR+CD3-CD56-CD19-PD-L1+	0.87 ± 0.19	0.78 ± 0.23	0.85

Supplementary Table 2a. Incidence of PD-L1$^+$CTCs amongPD-L1$^+$ or PD-L1$^-$ tumors in NSCLC patients.

	No of patients (%)	
	PD-L1$^+$ tumors	**PD-L1$^-$tumors**
PD-L1$^+$ CTCs	1 (10)	2 (20)
PD-L1$^-$CTCs	2 (20)	5 (50)

Supplementary Table 2b. PD-L1$^+$ CTCs andPD-L1$^+$ tumors in NSCLC patients.

Patients	PD-L1$^+$ CTCs	PD-L1$^+$ tumors
1	0	ND
2	1	0
3	0	ND
4	0	ND
5	8	ND
6	0	ND
7	1	0
8	0	ND
9	0	0
10	1	ND
11	1	ND
12	0	0
13	0	0
14	0	10
15	0	0
16	0	ND
17	0	ND
18	0	ND
19	0	ND
20	0	ND
21	0	ND
22	0	ND
23	0	ND
24	0	ND
25	0	ND
26	1	ND
27	1	ND
28	0	ND
29	0	ND
30	0	ND

ND: non-determined

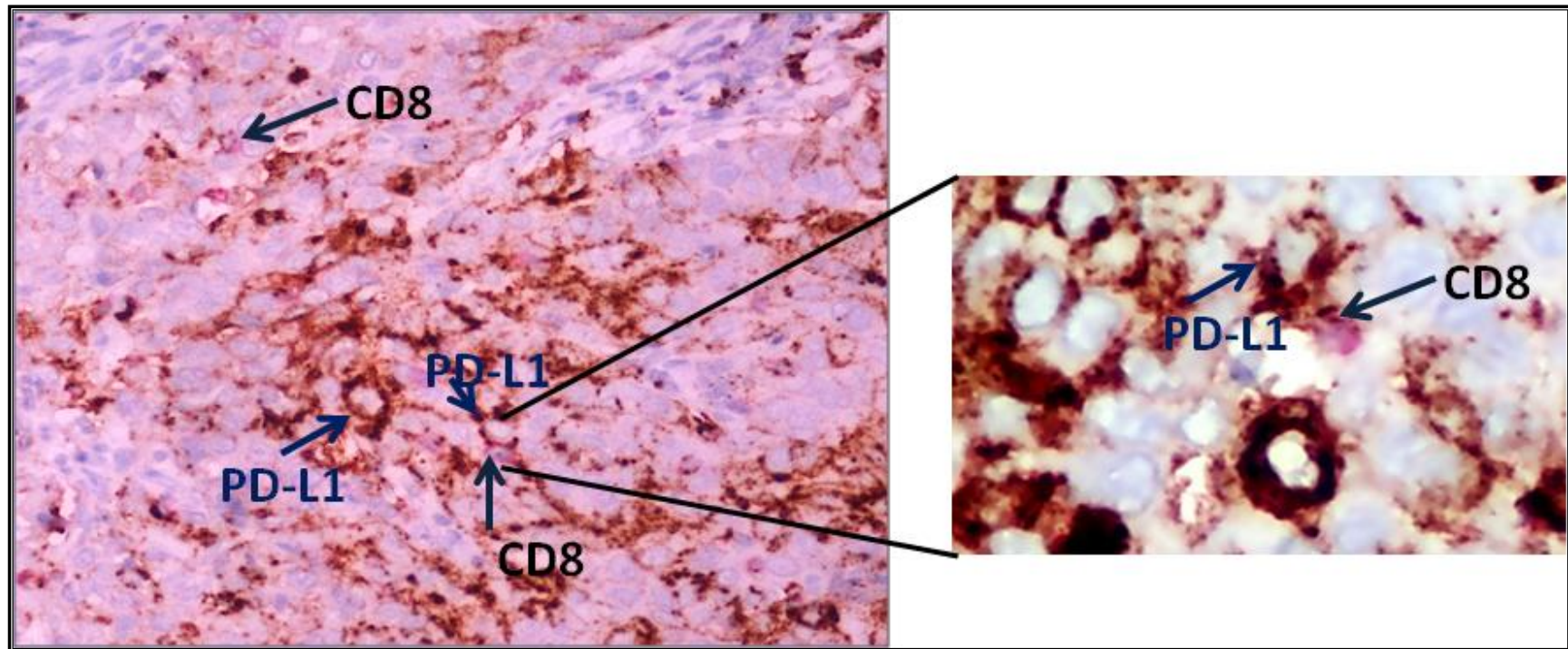**Supplementary Table 3. Association of Immune cells and clinical outcome of treatment-naive NSCLC patients.** N= number of patients, CI= confidence intervals, SQ=squamous, ND= not defined, DC=Dendritic cells

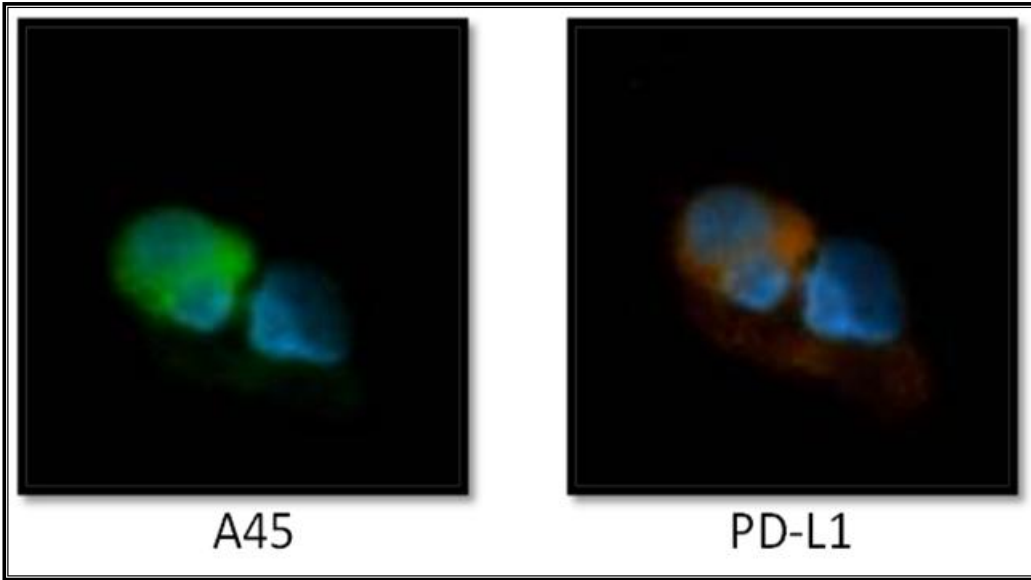|	PROGRESSION-FREE SURVIVAL				OVERALL SURVIVAL			
	n	Events	Months (95% CI)	p value	n	Events	Months (95% CI)	p value
Clinical parameters								
Age								
(≥ 68 vs <68)	18 vs 14	15 vs 12	3.7 (2.2-5.3) vs 4.3 (3.1-5.5)	0.59	**18 vs 14**	**10 vs 3**	**6.9 (4.1-9.6) vs 9.2 (7.6-10.9)**	**0.04**
Gender								
(Male vs Female)	31 vs 1	27 vs 0	ND	ND	31 vs 1	13 vs 0	ND	ND
Histology								
(Non-SQ vs SQ)	20 vs 12	17 vs 10	3.9 (2.5-5.2) vs 4.3 (2.7-5.9)	0.65	20 vs 12	9 vs 4	7.2 (5.4-8.9) vs 9.9 (6.7-13.2)	0.61
Stage								
(M1b vs non-M1b)	21 vs 11	17 vs 10	3.1 (2.3-3.9) vs 5.3 (3.4-7.3)	0.06	21 vs 11	9 vs 4	7.2 (5.2-9.1) vs 9.7 (6.4-13.1)	0.52
Immunological parameters (< vs ≥ mean)								
CD4$^+$ T cells	13 vs 19	12 vs 15	3.6 (1.9-5.2) vs 4.3 (2.9-5.5)	0.57	13 vs 19	7 vs 6	6.5 (4.2-8.7) vs 9.7 (7.0-12.4)	0.38
PD-1$^+$ CD4$^+$ T cells	16 vs 16	15 vs 12	4.1 (2.6-5.6) vs 4.1 (2.6-5.6)	0.91	16 vs 16	8 vs 5	7.1 (5.0-9.2) vs 9.5 (6.4-12.5)	0.60
PD-L1$^+$ CD4$^+$ T cells	19 vs 13	17 vs 10	3.9 (2.6-5.4) vs 4.2 (2.5-5.9)	0.73	19 vs 13	10 vs 3	7.1 (5.1-8.9) vs 10.9 (7.9-13.9)	0.32
CD8$^+$ T cells	15 vs 17	12 vs 15	4.8 (2.9-6.6) vs 3.3 (2.4-4.3)	0.16	15 vs 17	6 vs 7	9.2 (6.3-12.1) vs 7.1 (5.0-9.2)	0.89
PD-1$^+$ CD8$^+$ T cells	**20 vs 12**	**17 vs 10**	**4.8 (3.5-6.1) vs 2.8 (1.5-4.1)**	**0.02**	20 vs 12	7 vs 6	9.9 (7.5-12.3) vs 6.1 (3.6-8.5)	0.23
PD-L1$^+$CD8$^+$ T cells	18 vs 14	16 vs 11	4.7 (3.3-6.0) vs 3.1 (1.8-4.3)	0.23	18 vs 14	6 vs 7	10.0 (7.4-12.6) vs 4.9 (3.6-6.2)	0.19
B cells	19 vs 13	18 vs 9	3.4 (2.2-4.7) vs 4.9 (3.3-6.5)	0.20	19 vs 13	9 vs 4	7.0 (5.0-9.0) vs 9.2 (5.8-12.6)	0.52
PD-1$^+$ B cells	22 vs 10	20 vs 7	3.7 (2.5-4.8) vs 4.8 (2.8-6.8)	0.29	22 vs 10	9 vs 4	7.4 (5.6-9.4) vs 8.4 (4.7-12.2)	0.95
PD-L1$^+$ B cells	25 vs 7	24 vs 3	3.9 (2.8-4.9) vs 4.6 (2.2-6.9)	0.37	2 5 vs 7	10 vs 3	9.4 (7.2-11.5) vs 4.6 (2.2-7.0)	0.29
DC/monocytes	**11 vs 21**	**10 vs 17**	**3.3 (1.2-5.5) vs 4.4 (3.4-5.5)**	**0.05**	**11 vs 21**	**7 vs 6**	**5.3 (2.7-7.8) vs 10.4 (8.1-11.7)**	**0.04**
PD-1$^+$ DC/monocytes	17 vs 15	12 vs 15	3.8 (2.6-5.1) vs 4.1 (2.6-5.5)	0.98	17 vs 15	5 vs 8	8.2 (5.9-10.4) vs 8.6 (5.9-11.3)	0.68
PD-L1$^+$DC/monocytes	23 vs 9	21 vs 6	3.9 (2.7-5.1) vs 4.4 (2.1-6.7)	0.53	23 vs 9	9 vs 4	7.8 (5.9-9.6) vs 8.1 (4.1-12.0)	0.64

Supplementary Figures

Supplementary Figure 1. (A) Representative photomicrographs of immunohistochemical staining for PD-L1 and CD8^{+} TILs. Blue arrows indicate the positively PD-L1 stained cancer cells and black arrows the infiltrating CD8^{+} T cells. **(b)** Representative membrane spots from NSCLC patient stained for Cytokeratin (A45; green), PD-L1 (red) and DAPI.
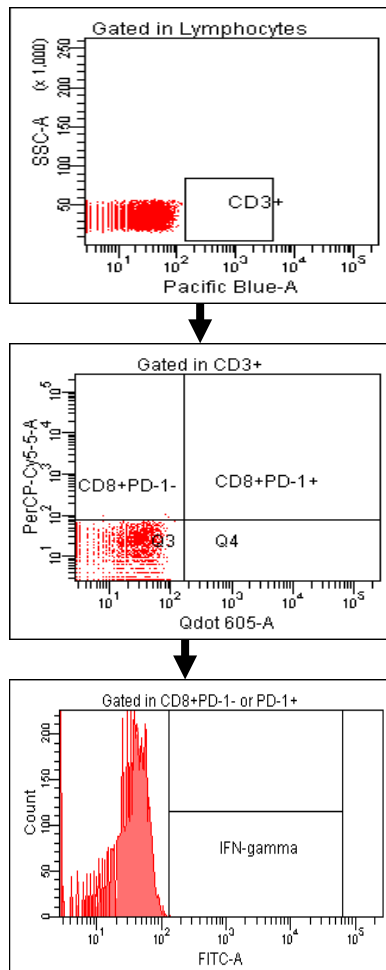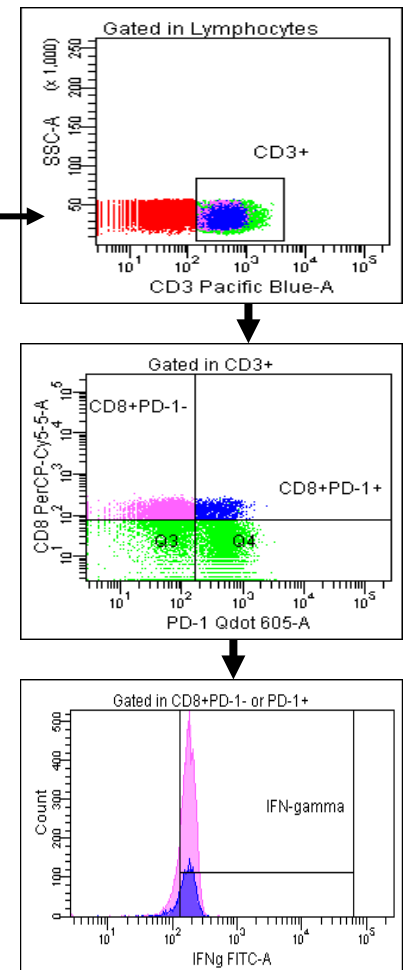
A

B

A45   PD-L1

Supplementary Figure 2. The functionality of CD8$^+$PD-1$^+$ T cells in NSCLC patients. Representative dot plots of flow cytometric analysis of IFN-γ and PD-1 gated in CD8$^+$ T cells. The gates for dot plots are presented on the top of each box. The positive expression of markers is compared to cells without antibody staining.

Supplementary Figure 3. Phenotypic analysis of (a) $CD4^+PD-1^+$ or $PD-L1^+$ T cells and $CD8^+PD-1^+$ or $PD-L1^+$ T cells,(b) $CD19^+PD-1^+$ or $PD-L1^+$ B cells and (c)$PD-1^+$ or $PD-L1^+$ DC/monocytes in NSCLC patients. Representative dot plots, as well as the gating strategy for identification and quantification of effector immune cells expressing PD-1 or PD-L1. Arrows indicate the sequence of gating. The gates for each dot plot are presented on the top of each box.
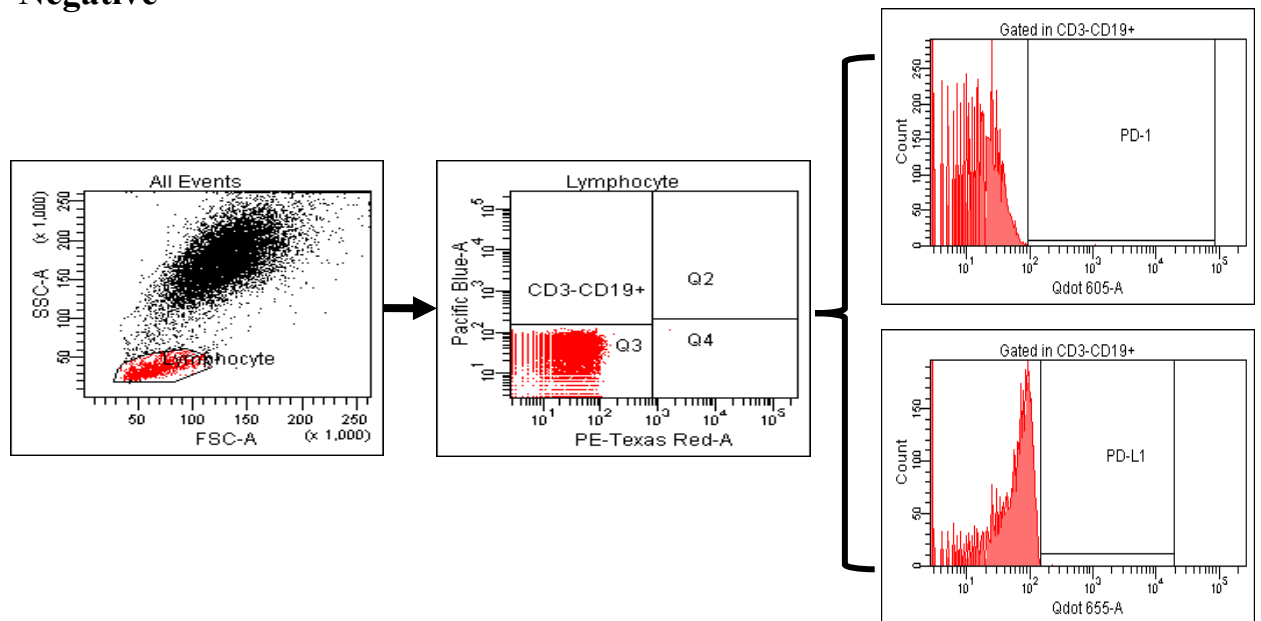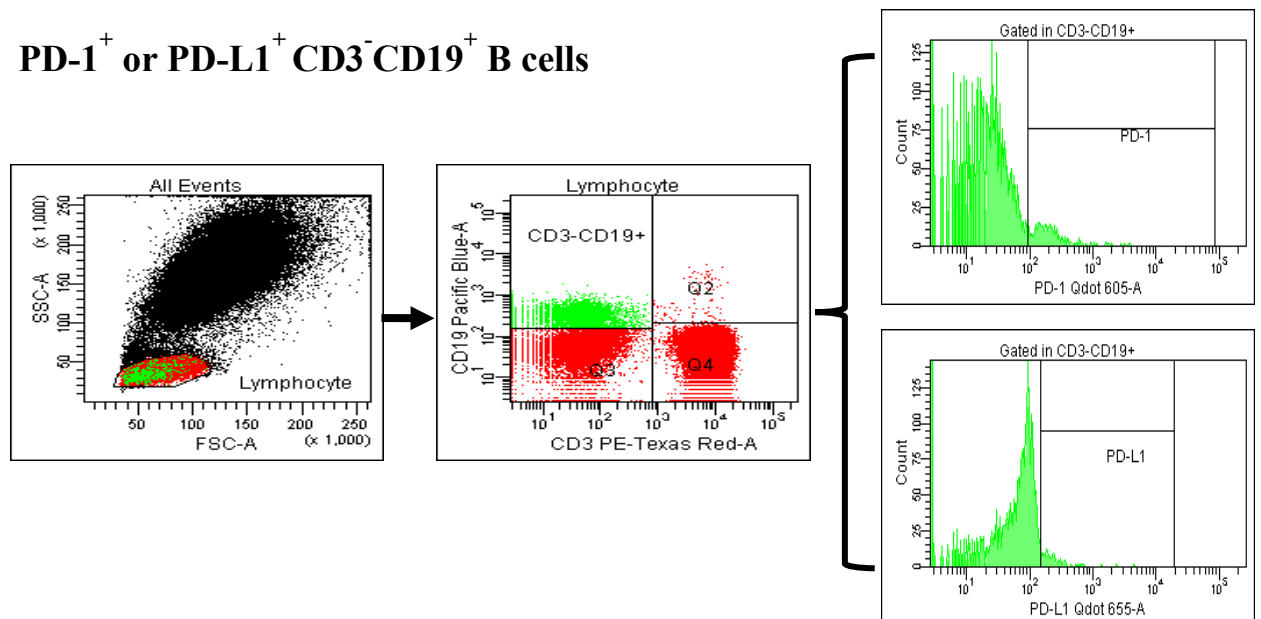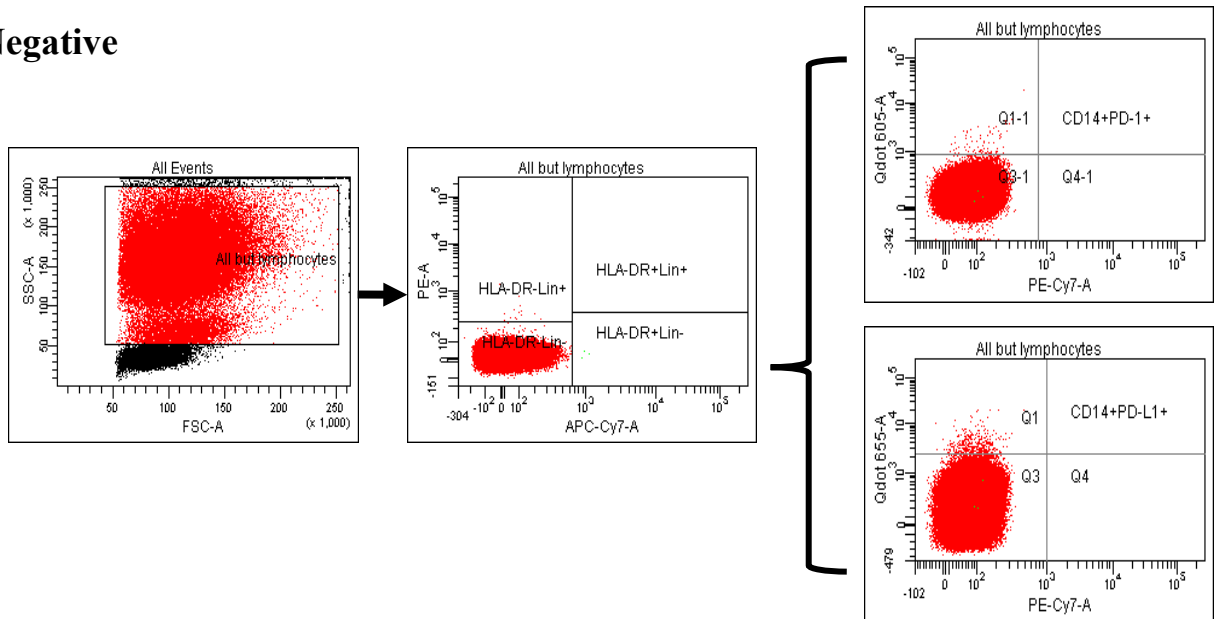
A

B

Negative

PD-1$^+$ or PD-L1$^+$ CD3$^-$CD19$^+$ B cells

C

Negative

PD-1$^+$ or PD-L1$^+$ DC/monocytes