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 |  |  |
|------------|-----------|--------------------|---|---|
| CD4+ T     | CD3−CD4+  | 47.01 ± 2.92       | 52.32 ± 4.02 | 0.28 |
| CD4+ T     | CD3−CD4+PD-1+ | 39.29 ± 2.69   | 42.30 ± 3.64 | 0.50 |
| CD4+ T     | 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 |
| CD8+ T     | CD3−CD8+PD-1+ | 17.17 ± 1.8     | 16.04 ± 2.9  | 0.73 |
| CD8+ T     | 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 |
| B cells    | CD3−CD19+PD-1+ | 1,11 ± 0.26     | 0.8846 ± 0.25 | 0.6425 |
| B cells    | 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 |
| DC/monocytes | CD14−HLA-DR+CD3−CD56−CD19−PD-1+ | 19.96 ± 2.22 | 15.40 ± 2.98 | 0.12 |
| DC/monocytes | 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 among PD-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 and PD-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

| Clinical parameters | PROGRESSION-FREE SURVIVAL | OVERALL SURVIVAL |
|---------------------|--------------------------|-----------------|
|                     | n | Events | Months (95% CI) | p value | n | Events | Months (95% CI) | p value |
| **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 | 25 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.
Supplementary Figure 2. The functionality of CD8\(^{+}\)PD-1\(^{+}\) T cells in NSCLC patients. Representative dot plots of flow cytometric analysis of IFN-\(\gamma\) 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.

**Negative control**

**PD-1\(^{+}\) or PD-1\(^{-}\)CD8\(^{+}\) T cells**
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 Negative

PD-1⁺ or PD-L1⁺ CD4⁺ or CD8⁺ T cells
B

Negative

PD-1⁺ or PD-L1⁺ CD3⁻CD19⁺ B cells
C

Negative

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