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Notes and Insights

Peripheral Polyfunctional PD1$^+$CD8$^+$ T cells demonstrated strong immune protection in non-small cell lung cancer

Surgical resection is the first treatment choice for early-stage non-small cell lung cancer (NSCLC) patients. Approximately 30% of patients eventually relapse [1]. Local immunological indicators, such as CD8$^+$ tumor-infiltrating T cells, are correlated with clinical outcomes [2–4]. Polyfunctional T cells, which simultaneously secrete multiple cytokines, exhibit strong immune protective capabilities [5–7]. Herein, we assessed whether peripheral polyfunctional PD1$^+$CD8$^+$ T cells are

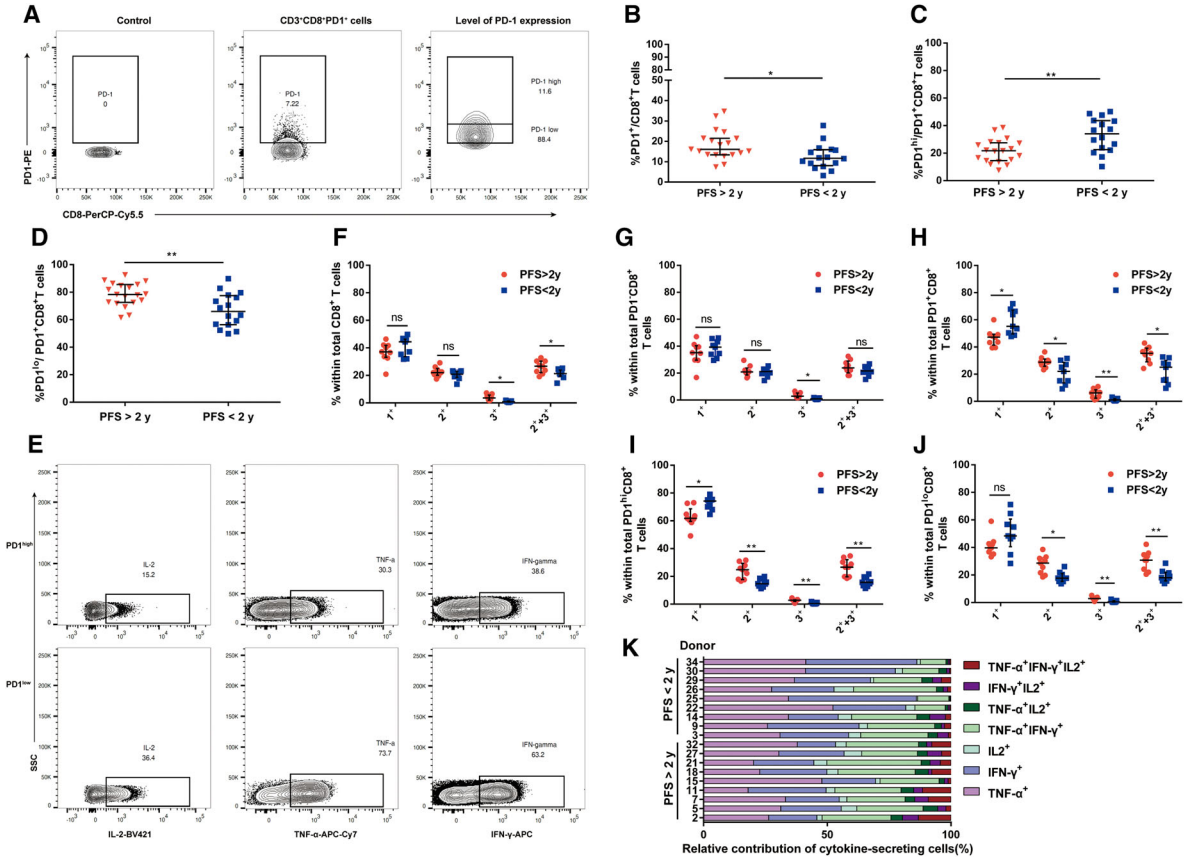

Figure 1. Frequencies and polyfunctionality of peripheral CD8$^+$ T-cell subsets in early-stage NSCLC patients. (A) Representative FACS data of peripheral PD1-expressing CD8$^+$ T cells. (B–D) Proportions of PD1$^+$, PD1^{hi}, and PD1^{lo} cells among CD8$^+$ T-cell subsets in patients with PFS > 2 years (PFS>2y, $n = 19$) and PFS < 2 years (PFS<2y, $n = 16$) after surgery. PBMCs were collected 2 weeks before surgery, stained with relevant antibodies, and detected using flow cytometry. (E) Representative FACS data of intracellular cytokine release by PD1^{hi}CD8$^+$ and PD1^{lo}CD8$^+$ T cells. (F-J) Frequencies of cytokine-secreting cells among CD8$^+$ T cell subsets in two patient groups ($n = 9$/group). 1^+, 2^+, 3^+, $2^+ + 3^+$: cells simultaneously secreting one, two, three or at least two cytokines, respectively. (K) Multicytokine-secreting distribution of PD1$^+$CD8$^+$ T cells in two patient groups ($n = 9$/group). PBMCs were stimulated with ionomycin/PMA for 6 h, then stained for cytokine production and detected using flow cytometry (E–K). Each dot shows the mean of two technical repeats of each sample (one of two independent experiments). Error bars represent the median with interquartile range. $^*p < 0.05$, $^{**}p < 0.01$, ns: not significant (t-test).

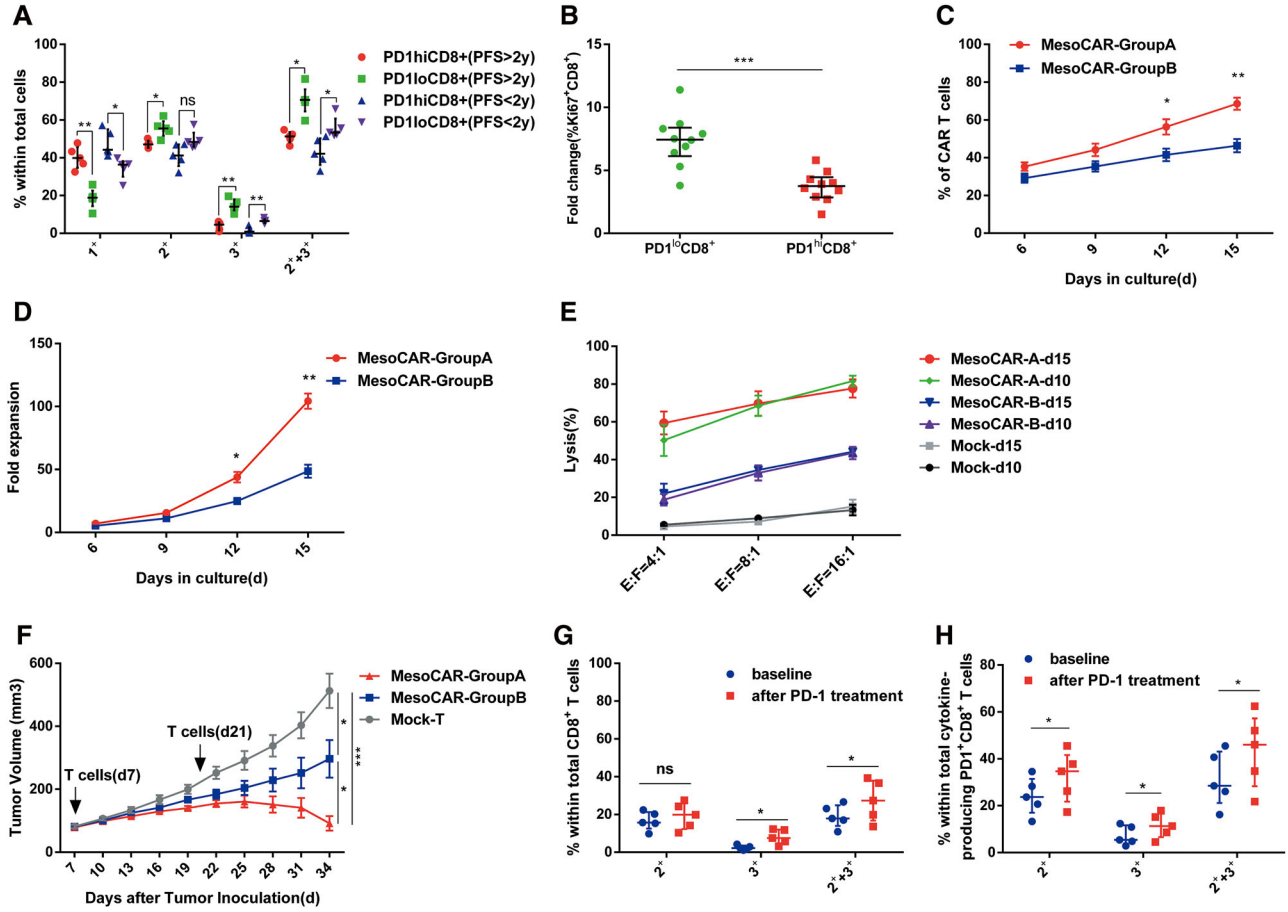

Figure 2. Cytotoxic capacity of polyfunctional PD1$^+$CD8$^+$ T cells. (A) Polyfunctionality of PD1^{hi}CD8$^+$ and PD1^{lo}CD8$^+$ T cells. Cells were sorted from PBMCs of two patient groups ($n = 5$/group) and stimulated with ionomycin/PMA for 6 h, then stained for cytokine production and detected using flow cytometry. (B) In vitro proliferative capacity of sorted PD1^{hi}CD8$^+$ and PD1^{lo}CD8$^+$ T cells from PBMCs of patients ($n = 5$/group). Cells were stimulated with CD3/CD28 beads and IL-2 for 5 days; then, cells were stained with relevant antibodies and measured using flow cytometry. The fold change of Ki-67$^+$ cells was determined according to baseline measurements. (C and D) Percentages of mesothelin-positive cells and expansion rate of MesoCAR-T cells during in vitro culture with CD3/CD28 beads and IL-2. MesoCAR-T cells were manufactured with T cells of groupA ($2^+ + 3^+ > 50\%$, $n = 3$) and groupB ($2^+ + 3^+ < 30\%$, $n = 3$), all collected from patients with PFS > 2 years. (E) Killing ability of MesoCAR-GroupA and MesoCAR-GroupB T cells. T cells, which collected on the 10th and 15th day during in vitro culture, were cocultured with MSTO-MSLN$^+$ffLuc$^+$ cells for 24 h at different E:T ratios, followed by luciferase killing assay. (F) NSG mice ($n = 4$ mice/group) were subcutaneously injected with 6×10^5 MSTO-MSLN$^+$ffLuc$^+$ cells and 1×10^6 CAR-T cells or mock T cells were infused via tail-vein injection on the 7th and 21st day after tumor inoculation. Tumor size was calculated using a vernier caliper twice a week. (G and H) Polyfunctionality of peripheral CD8$^+$ and PD1$^+$CD8$^+$ T cells of advanced NSCLC patients before and after anti-PD-1 therapy. PBMCs were collected 2 weeks before (baseline) and 90 days after anti-PD-1 therapy, stimulated with ionomycin/PMA for 6 h, followed by antibody staining and flow cytometry detection. Each dot shows the mean of two technical replicates of each sample (A and B, G and H, one of two independent experiments), error bars represent the median with interquartile range. Data from (C–F) are shown as mean ± SEM (one of three independent experiments), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant (*t*-test for [A-D, G-H], one-way ANOVA for [F]).

correlated with progression-free survival (PFS) in early-stage NSCLC patients after surgery.

We recruited 35 early-stage NSCLC patients, including 19 with PFS > 2 years and 16 with PFS < 2 years since undergoing surgery. We collected peripheral blood mononuclear cells (PBMCs) 2 weeks before surgery. PD1$^+$CD8$^+$ T cells, examined by flow cytometry, showed higher frequencies in patients with PFS > 2 years (Fig. 1B). Furthermore, the frequency of PD1^{hi}CD8$^+$ T cells was higher in patients with PFS < 2 years, whereas the proportion of PD1^{lo}CD8$^+$ T cells was higher in patients with PFS > 2 years (Fig. 1C and D). These data can facilitate the identification of correlations between peripheral PD1$^+$CD8$^+$ T cells and PFS in cancer patients.

To investigate whether the polyfunctionality of PD1$^+$CD8$^+$ T cells showed differences between the two patient groups, we performed multiparametric flow cytometry to identify intracellular cytokines (IFN-γ, TNF-α, and IL-2) secreted following ionomycin/PMA stimulation. The frequency of polyfunctional cells among the total PD1$^+$CD8$^+$ T cells was much higher in patients with PFS > 2 years (Fig. 1H and K). The similar results were observed in PD1^{hi}CD8$^+$ and PD1^{lo}CD8$^+$ T cells (Fig. 1I and J). Furthermore, the percentage of polyfunctional cells among sorted PD1^{lo}CD8$^+$ T cells was much higher than among sorted PD1^{hi}CD8$^+$ T cells in both patient groups (Fig. 2A). Compared with PD1^{hi}CD8$^+$ T cells, PD1^{lo}CD8$^+$ T cells showed stronger

proliferative capacity when stimulated by CD3/CD28 beads in vitro (Fig. 2B). These data suggest that higher percentages of polyfunctional $PD1^+CD8^+$ T cells lead to better PFS in early-stage NSCLC patients after surgery.

Mesothelin-specific CAR-T cells were manufactured with T cells from the PFS > 2 years group to assess correlations between the polyfunctionality and cytotoxicity of $PD1^+CD8^+$ T cells. The frequencies of polyfunctional cells among total cytokine-producing $PD1^+CD8^+$ T cells of >50% and <30% were defined as MesoCAR-GroupA and MesoCAR-GroupB, respectively. T cells in MesoCAR-GroupA exhibited stronger proliferative capacity during in vitro culture (Fig. 2C and D). CAR-T cells and mesothelin stable-expression target cells (MSTO-$MSLN^+ffLuc^+$) were cocultured in vitro at different E:T ratios. MesoCAR-GroupA T cells showed stronger cytotoxic capacity than MesoCAR-GroupB T cells (Fig. 2E). An NSG mouse model stably expressing mesothelin was established by subcutaneous injection of MSTO-$MSLN^+ffLuc^+$ cells. Then, MesoCAR-T cells and mock T cells were infused into the mice on the seventh day (D7) and D21 via tail vein injection. Both MesoCAR-GroupA and MesoCAR-GroupB T cells exhibited tumor control compared with mock T cells (Fig. 2F). However, the tumor burden was significantly lower after the second infusion of MesoCAR-GroupA T cells, indicating a more effective antitumor activity from MesoCAR-GroupA T cells than MesoCAR-GroupB T cells.

To determine the effect of PD-1 inhibitor treatment on the polyfunctionality of peripheral $PD1^+CD8^+$ T cells, we recruited five advanced NSCLC patients who previously received second-line systemic chemotherapy and collected PBMCs 2 weeks prior and 90 days after PD-1 inhibitor treatment. The multicytokine-producing capacity of $CD8^+$ and $PD1^+CD8^+$ T cells was detected using flow cytometry. The polyfunctionality of peripheral $CD8^+$ and $PD1^+CD8^+$ T cells was significantly rescued after anti-PD-1 therapy (Fig. 2G and H).

In summary, we demonstrated a positive correlation between peripheral polyfunctional $PD1^+CD8^+$ T cells and PFS in early-stage NSCLC patients after surgery. Furthermore, we found that polyfunctional $PD1^+CD8^+$ T cells exhibited effective cytotoxic capacity in vitro and in vivo and that the polyfunctionality of $PD1^+CD8^+$ T cells increased after anti-PD-1 treatment. Therefore, this study suggested that CAR-T therapy is a potentially effective treatment option for patients who have already been treated with PD-1 antibody.

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