The combination of a PTP1B inhibitor, TNFR2 blocker, and PD-1 antibody suppresses the progression of non-small cell lung cancer tumors by enhancing immunocompetence

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Abstract. Lung cancer is increasingly recognized as a leading cause of cancer-related mortality. Immunotherapy has emerged as a promising therapeutic approach for lung cancer, particularly non-small cell lung cancer (NSCLC). Nonetheless, the response rate to programmed cell death 1 (PD-1) inhibitors remains less than optimal. It has been suggested that protein tyrosine phosphatase 1B (PTP1B) plays a crucial role in the development and progression of cancer by facilitating T cell expansion and cytotoxicity. Our previous research demonstrated that the combination of tumor necrosis factor receptor 2 (TNFR2) with immune activity treatments synergistically suppresses tumor growth. This insight led to exploring the efficacy of a combined treatment strategy involving PD-1 inhibitors, PTP1B inhibitors and TNFR2 antibodies (triple therapy) in NSCLC. In this context, the therapeutic effectiveness of these combination immunotherapies was validated in mouse models with NSCLC by analyzing the expansion and function of immune cells, thereby assessing their impact on tumor growth. The results indicated that inhibiting PTP1B decreases the expression of PD-L1 and TNFR2 on LLC cells, along with an increase in the proportion of $CD4^+$T and $CD8^+$T cells. Compared with other treatment groups, the triple therapy significantly reduced tumor volume in mice with NSCLC and extended their survival. Moreover, this combination therapy altered the distribution of myeloid-derived suppressor cells, dendritic cells, B cells and M1 macrophages, while increasing the proportion of $CD8^+$T cells and reducing the proportion of Treg cells in the spleens, lymph nodes, and tumors of NSCLC models. The triple therapy also resulted in a decrease in PD-L1, PTP1B and TNFR2 expression within NSCLC tumor tissues in mice. Overall, the triple therapy effectively suppressed tumor growth and improved outcomes in mice with NSCLC by modulating immune cell distribution and reducing levels of target immune proteins.

Introduction

As of 2020, lung cancer was responsible for the deaths of ~1.8 million individuals worldwide, making it the leading cause of cancer mortality. Non-small cell lung cancer (NSCLC) constitutes the majority of these cases, accounting for ~85% (1,2). The surgical resection, despite its inherent risks, has unequivocally exhibited a remarkable decline in mortality rates among patients afflicted with operable early-stage and locally advanced NSCLC; 25-70% of surgical patients eventually relapse, yielding a worse prognosis (3). The current landscape of advanced lung cancer treatment has witnessed the ascendancy of immunotherapy as the foremost therapeutic modality (4). Among them, the application of immune checkpoint blockade has demonstrated remarkable efficacy in improving survival outcomes; however, no response cases still prevail among a substantial proportion of patients undergoing treatment (5,6). The mechanisms by which the body resists immunotherapy and the development of strategies to make

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advanced and metastatic disease manageable are urgently needed to be explored.

Blockers targeting programmed cell death protein 1 (PD-1) and its ligand PD-L1 have been granted approval for the treatment of patients with NSCLC, owing to their remarkable efficacy in improving overall survival (7). The administration of PD-1 or PD-L1 antibodies rescues T cells from a state of exhaustion, reinvigorating the immune response against cancer cells (8). However, some patients could not significantly improve their survival rate after treatment with PD-1 blockers, and radiotherapy also did not increase responses to combination with PD-1/PD-L1 therapy (9). The risk of mortality was significantly diminished in both PD-L1 positive and PD-L1 negative patients when subjected to PD-1/PD-L1 blockade as compared with conventional therapy in clinical trials (10). Notably, the combination therapy involving PD-1/PD-L1 inhibitors has exhibited a remarkable augmentation in clinical outcomes for patients with NSCLC and brain metastases (11). Therefore, combining PD-1/PD-L1 inhibitors with co-stimulatory molecule agonists can synergistically enhance multiple processes in the cancer-immunity cycle and improve the tumor microenvironment by reducing immunosuppression (8).

The non-transmembrane enzyme, protein tyrosine phosphatase 1B (PTP1B), is widely acknowledged as a negative regulator of metabolic signaling pathways (12). Recently, a survival analysis revealed a significant upregulation of PTP1B in the majority of tumor tissues, which exhibited a strong association with an unfavorable prognosis (13). The PTP1B inhibitor MSI-1436 demonstrated remarkable drug sensitivity by significantly suppressing tumor cell viability, thereby suggesting its potential to augment the efficacy of PD-1 inhibitors across diverse cancer types (13). Moreover, the PTPN1 gene, encoding for PTP1B, serves as a negative regulator in various cytokine signaling pathways and T cell receptor signaling (14). PTP1B was identified a novel intracellular checkpoint that exert the function of modulating immune cell-tumor cell interaction as a tumor suppressing factor (15). The inhibition of PTP1B can potentiate the expansion and cytotoxicity of antigen-induced $CD8^+$ T cells, enhance the efficacy of PD-1 blockade and impede tumor progression (15,16). The findings suggest that PTP1B plays a crucial role in the cancer tumor microenvironment, but the exceptional treatment strategy and modulation mechanism in NSCLC immunotherapy remain unclear. A previous research by the authors has revealed a remarkable surge in the infiltration of cytotoxic T lymphocytes (CTLs) within both the tumor microenvironment and spleen subsequent to anti-TNFR2 immunotherapy (17). The simultaneous induction of CTLs and modulation of the tumor's immunosuppressive microenvironment synergistically culminatea in a curative efficacy against multiple malignancies (18). Therefore, the modulation of PTP1B and TNFR2 has the potential to augment the sensitivity of PD-1 blockade, amplify its antitumor efficacy, and protract patient survival by orchestrating dynamic alterations within the tumor microenvironment. Notably, PTP1B emerges as a pivotal target in relation to tumor immunotherapy.

In the present study, the objective was to investigate a more effective combination immunotherapy approach involving anti-PTP1B, anti-PD-1 and anti-TNFR2 for NSCLC while concurrently elucidating the contributions of multiple immune cells, thereby providing innovative targeted strategies for identifying patients with NSCLC.

Materials and methods

Animals and tumor models. Male C57BL/6 wild-type mice (aged 6-8 weeks) were procured from Spaefer Biotechnology Co, Ltd. The animal study was ethically approved (approval no. 2305196) by the Animal Research Ethics Committee at Guizhou University [Laboratory Animal License Number: SCXK (Guizhou) 2023-0002; Guiyang, China]. The mice were housed in SPF under standard conditions (22±2˚C, 50-70% humidity) and maintained on a 12-h light/dark cycle (light on 7:00 a.m.) with food and water available ad libitum. The mice were subcutaneously injected with 0.1 ml of PBS containing Lewis lung cancer cells (LLC, $2x10^6$/ml) in the unilateral flank (right side) to establish tumor models. Drug or administration interventions were initiated once the tumor diameter reached a pre-determined range of 6-8 mm (Fig. 1A-C). Each experimental group consisted of 5 mice. Additionally, to evaluate sustained immunocompetence induced by these immunotherapies in mice, an identical treatment regimen on a separate batch of mice was employed with the same number to assess their survival curve, as shown as Fig. 1D.

Combined immunotherapy. To evaluate the effect of immunotherapy in the lung cancer-bearing mice, the mice were randomized into different groups: (i) PBS group: intraperitoneal (i.p.) injection of PBS (Bio X Cell, 200 μl/mouse); (ii) i.p. injection of anti-PD-1 (cat. no. HY-P99144; MedChemExpress, 200 μg/200 μl/mouse); (iii) i.p. injection of anti-PD-1 (200 μg/100 μl/mouse) and i.p. injection of anti-TNFR2 (cat. no. HY-12219A, Bio X Cell, 100 μg/mouse); (iv) i.p. injection of anti-PD-1 (200 μg/100 μl/mouse) and injection of anti-PTP1B (MSI-1436; cat. no. HY-12219A; MedChemExpress; 100 μg/100 μl/mouse); and (v) i.p. injection of anti-PD-1 (200 μg/100 μl/mouse), anti-PTP1B (100 μg/100 μl/mouse) and anti-TNFR2 (100 μg/100 μl/mouse) (triple therapy). Intraperitoneal injections of anti-PD-1 and anti-TNFR2 were administered every 4 days for 2 weeks, while anti-PTP1B was injected once every 3 days for 2 weeks. Tumor size and body weight of mice were monitored every 3 days, with humane endpoints set at a tumor diameter >2,000 mm. Due to the weight loss often induced by immunotherapy mouse weights were rigorously monitored to ensure that any decrease did not exceed 20%, thereby mitigating potential suffering. The mice were euthanized through an overdose of sodium pentobarbital anesthesia (100 mg/kg, i.p.). The experiments adhered to institutional guidelines and ethical standards in relation to euthanasia and death verification. Upon observing indicators such as the absence of respiration, lack of cardiac activity, unresponsiveness to stimuli, muscular rigidity, and cyanosis without any signs of life, the occurrence of death was confirmed. These conditions were observed initially 5-10 min after injection and then confirmed again 30 min later.

Cell culture. The lung adenocarcinoma LLC cells (obtained from the Zhongqiao Xinzhou Biotechnology Co., Ltd. (cat. no. ZQ0203) were utilized for the experiments. The cells

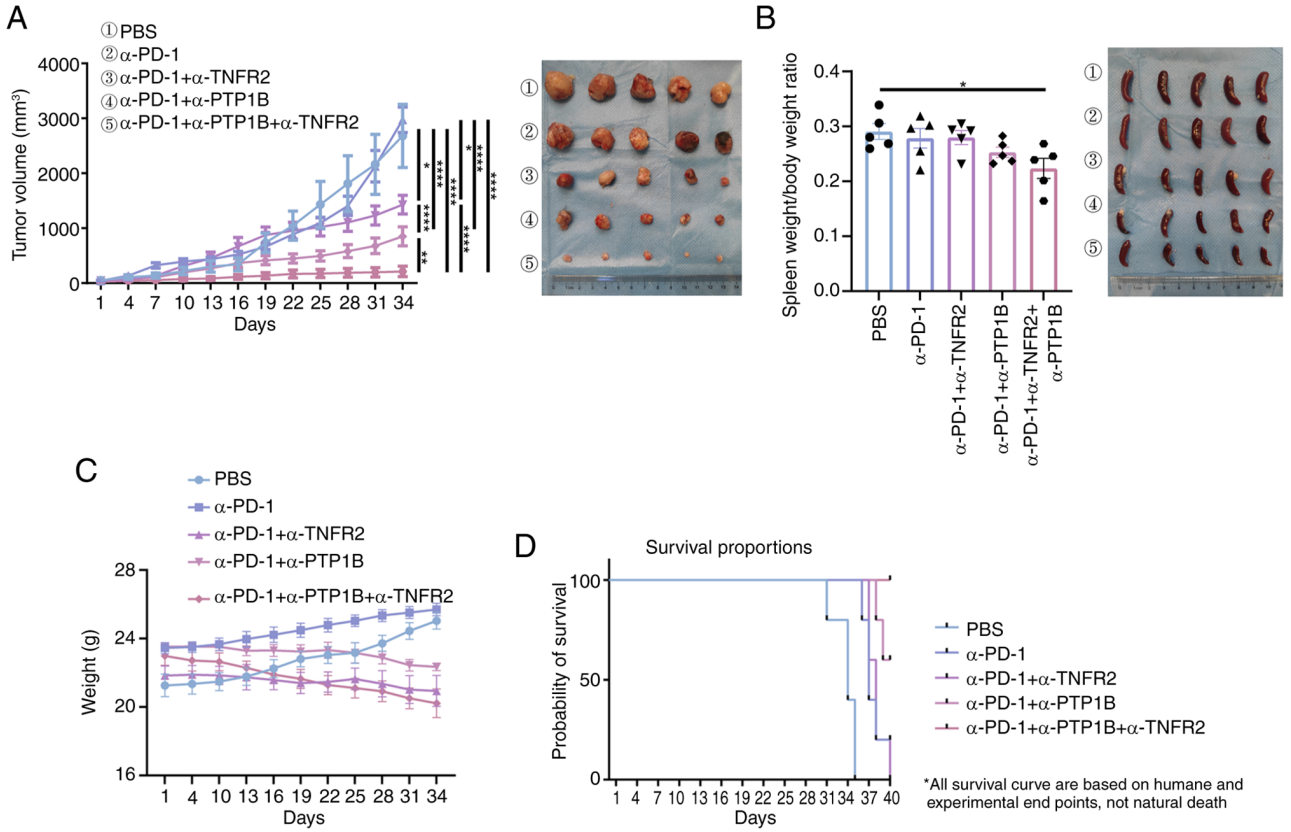

Figure 1. Combination immunotherapy effectively inhibits the growth of non-small cell lung cancer tumors. (A) The experimental design is illustrated in a schematic diagram. Tumor volume curves were presented for each group, accompanied by images showcasing the tumors (on the right). (B) The ratio of spleen/body weight was measured for each group, and images of spleens were shown (on the right). (C) The mice weights were evaluated for each group. (D) The survival rates were assessed for each group. The differences in tumor volume were analyzed using one-way ANOVA. The survival proportions were compared using the log-rank test. The data were presented as the mean ± SEM. *P<0.05, **P<0.01 and ****P<0.0001. PTP1B, protein tyrosine phosphatase 1B; PD-1, programmed cell death 1; TNFR2, tumor necrosis factor receptor 2.

were cultured in complete DMEM medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The LLC cells were maintained at a temperature of 37˚C in a humidified incubator with a gas mixture consisting of 5% CO_2 and 95% air.

Flow cytometry. To discern the infiltration of distinct leukocyte subsets in tumor tissues, spleen and lymph nodes, the antibodies used for flow staining diluted at 1:100 in single-cell suspensions; all the single-cell suspensions were adorned with rat anti-mouse CD45 conjugated to BV510 (BD Biosciences; clone 30-F11), hamster anti-mouse CD3 conjugated to BV605 (BD Biosciences; clone 145-2C11), rat anti-mouse CD4 conjugated to FITC (BD Biosciences; clone GK1.5), rat anti-mouse CD8 conjugated to Percp cy5.5 (BD Biosciences; clone 53-6.7) and rat anti-mouse CD25 conjugated to APC (BD Biosciences; clone PC61), rat anti-mouse IFN-gamma conjugated to PE (BD Biosciences; clone XMG1.2), rat anti-mouse Foxp3 conjugated to PE (BD Biosciences; clone R16-715), rat anti-mouse CD16/32 (BD Biosciences; clone 93), AR700-anti-Dead/Live (BD Biosciences; cat. no. 564997) at 4˚C for 30 min. After undergoing a meticulous triple washing process with FACS buffer, the data obtained from the stained samples was acquired using the state-of-the-art flow cytometer (BD FACSCelesta™; BD Biosciences). Cell debris was excluded based on forward and side scatter gating, and viable cells were analyzed using live/dead staining antibodies. The data analyses were conducted utilizing the FlowJo software (v10.8.1; FlowJo LLC), which enabled precise discrimination between cell populations.

Immunohistochemistry. The mouse tumor tissues were fixed in formalin, embedded in paraffin, cut into 4-μm sections, dewaxed with xylene, and rehydrated using a gradient of alcohol. After washing with distilled water, heat-induced antigen retrieval was performed using the decloaking chamber with low pH (pH 6.0) citrate buffer (10%, Thermo Fisher Scientific, Inc., cat. no. J61249) at 95˚C for 20 min, followed by cooling to room temperature for 15 min. The slides were then rinsed three times in PBS (pH 7.4) on a decolorizing shaker for a duration of 5 min per rinse before being blocked with a solution of 5% BSA (Sigma-Aldrich; Merck KGaA; cat. no. 9048-46-8) at room temperature for 1 h. After undergoing natural cooling, the slides were meticulously rinsed thrice in PBS (pH 7.4) on a decolorizing shaker for a duration of 5 min per rinse. Subsequently, the sections were treated with a 3% hydrogen peroxide solution and subsequently underwent three rounds of washing in PBS (pH 7.4) on a decolorizing shaking table for 5 min each time. Then, at room temperature, the tissue was covered with 3% BSA for 30 min. Primary antibodies including rabbit anti-PD-L1 (Absin; cat. no. abs136046;

1:200), anti-TNFR2 (Affinity Biosciences; cat. no. AF0364; 1:200) and anti-PTP1B (Absin; cat. no. abs131747; 1:200) were applied to the section in PBS at a specific ratio and incubated overnight at 4˚C in a humidified chamber. After being washed again in PBS on a shaking platform three times for 5 min each time, the sections were incubated with Goat anti-Rabbit HRP-labeled antibodies (Thermo Fisher Scientific, Inc.; cat. no. 31460; 1:1,000) at room temperature for 50 min. After undergoing an additional round of gentle agitation in PBS for three consecutive intervals of 5 min each, a freshly prepared peroxidase substrate solution DAB (Thermo Fisher Scientific, Inc.; cat. no. 34065) color-developing agent was introduced to facilitate the visualization of positive staining by a light microscope (BX53; Olympus Corporation).

Statistical analysis. Unless otherwise specified, all experiments were performed at least three times. The statistical analysis was conducted employing the one-way ANOVA test for multiple groups and the Scheffe post hoc test. The assessment of survival advantage was performed using the log-rank test. Statistical analyses were executed using GraphPad Prism 10 software (Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Combination immunotherapy suppresses NSCLC tumor growth in mice. To evaluate the potential synergistic impact of combining anti-PD-1 or anti-TNFR2 with anti-PTP1B on enhancing the antitumor immune response mediated in NSCLC, murine models (C57BL/6) were utilized to subcutaneously implant LLC lung cancer cells and the effect on tumor volume was assessed based on group allocation and treatment regimen. The results demonstrated that monotherapy with anti-PD-1 had minimal impact on tumor volume in murine models of lung cancer. Treatment with a combination of anti-PD-1 and either anti-PTP1B or anti-TNFR2 in mice resulted in a significant reduction in tumor volume. Moreover, the combination of anti-PD-1 and anti-PTP1B exhibited superior efficacy compared with the combination of anti-PD-1 with only anti-PTP1B. Notably, the triple therapy consisting of anti-PD-1, anti-PTP1B and anti-TNFR2 demonstrated significantly smaller tumor volumes compared with the PBS control group or any single or double therapy groups, as revealed in Fig. 1A. The ratio of spleen to body weight had a decrease in the triple therapy compared with PBS group mice (Fig. 1B). This suggests that the triple therapy may significantly enhance the systemic immune response, potentially boosting the immune activity against tumors in spleen, while the PBS group resulted in splenomegaly or inflammation. No significant weight loss was observed with immunotherapy in all groups (Fig. 1C). Thus, the triple therapy demonstrated superior efficacy compared with all other treatments. Meanwhile, humane euthanasia was performed as follows: all mice in the PBS group, PD-1 group and PD-1 + TNFR2 group were euthanized (n=5 per group; 100% mortality rate). In the PD-1 + PTP1B group, two out of five mice were euthanized (40% mortality rate within this group). Notably, triple therapy demonstrated remarkable efficacy with a 100% survival rate at the end of a 40-day treatment period (Fig. 1D). The findings indicated that the triple therapy significantly inhibits tumor growth and prolongs survival in mice with NSCLC tumor, while exhibiting negligible toxic side effects, rendering it a safe and efficacious immunotherapeutic approach for this malignancy.

Triple therapy significantly induces the alterations of the immune cells' population distribution. To explore whether combination therapies can elicit multiple immune cells effects on the tumor growth, the expression of immune cells NSCLC tumor in mice was initially assessed. It was observed that the repression of tumor growth was accompanied by alterations in the abundance of the tumor immune microenvironment (TIME). Compared with the PBS group, both the α-PD-1 + α-TNFR2 group and α-PD-1 + α-PTP1B group exhibited a decrease in monocytic myeloid-derived suppressor cells (MDSCs), which was immunosuppressive, the α-PD-1 group did not exhibit any statistically significant differences. As demonstrated in Fig. 2A, the triple therapy was accompanied by the reduce of MDSCs, which suggested that it may dampen the immune response or alleviate immunosuppression. By contrast, the triple therapy significantly augmented B cell recruitment, amplifying the impact of recruitment in the combination group of anti-PD-1 with anti-PTP1B (Fig. 2B). In the tumor-associated macrophages (TAMs), although the anti-PD-1 + anti-TNFR2 group, the anti-PD-1 + anti-PTP1B group and the triple therapy group exhibited an increasing trend, the proportion of M1 TAMs in the PBS group, the anti-PD-1 group and the anti-PD-1 + anti-TNFR2 group was lower compared with that in the triple therapy group. Particularly, the level of the anti-tumorigenic $CD86^+$M1 TAMs was found to be significantly higher in the triple therapy group compared with the anti-PD-1 + anti-TNFR2 group (Fig. 2C). Additionally, the triple therapy significantly expanded the percentage of antigen-presenting dendritic cells (DCs) (Fig. 2D), which promotes antitumor immunity, enhanced by the triple therapy. Notably, although the combination of anti-PD-1 with either anti-TNFR2 or anti-PTP1B slightly suppressed MDSCs and promoted macrophages, compared with these therapies, the triple therapy exhibited optimal efficacy for immune activation in NSCLC tumors in mice.

Triple therapy distinctly increases the population of $CD8^+$T cells and the expression of IFN-γ. Although nearly all immune cells have the potential to influence immune compartments in order to enhance antitumor immunity, focus was directed towards $CD8^+$T cells due to their predominant representation as tumor-infiltrating lymphocytes within cancerous tumors. Flow cytometric analysis of mouse tumors, spleens and draining lymph nodes was conducted for each immunotherapy group. The findings unveiled a remarkable surge in the abundance of $CD8^+$T cells within tumors subjected to the combination therapy of anti-PD-1 and anti-PTP1B, as well as the triple therapy groups. Notably, compared with PBS, anti-PD-1 monotherapy and dual therapy groups, triple therapy exhibited a significant enhancement in the proportion of $CD8^+$T cells (Fig. 3A). The triple therapy elicited a significant expansion of $CD8^+$T cells in the spleen, surpassing the combination of anti-PD-1 and anti-PTP1B or monotherapy with anti-PD-1 alone (Fig. 3B). Moreover, combined with α-TNFR2 or α-PTP1B augmented the expansion of $CD8^+$T

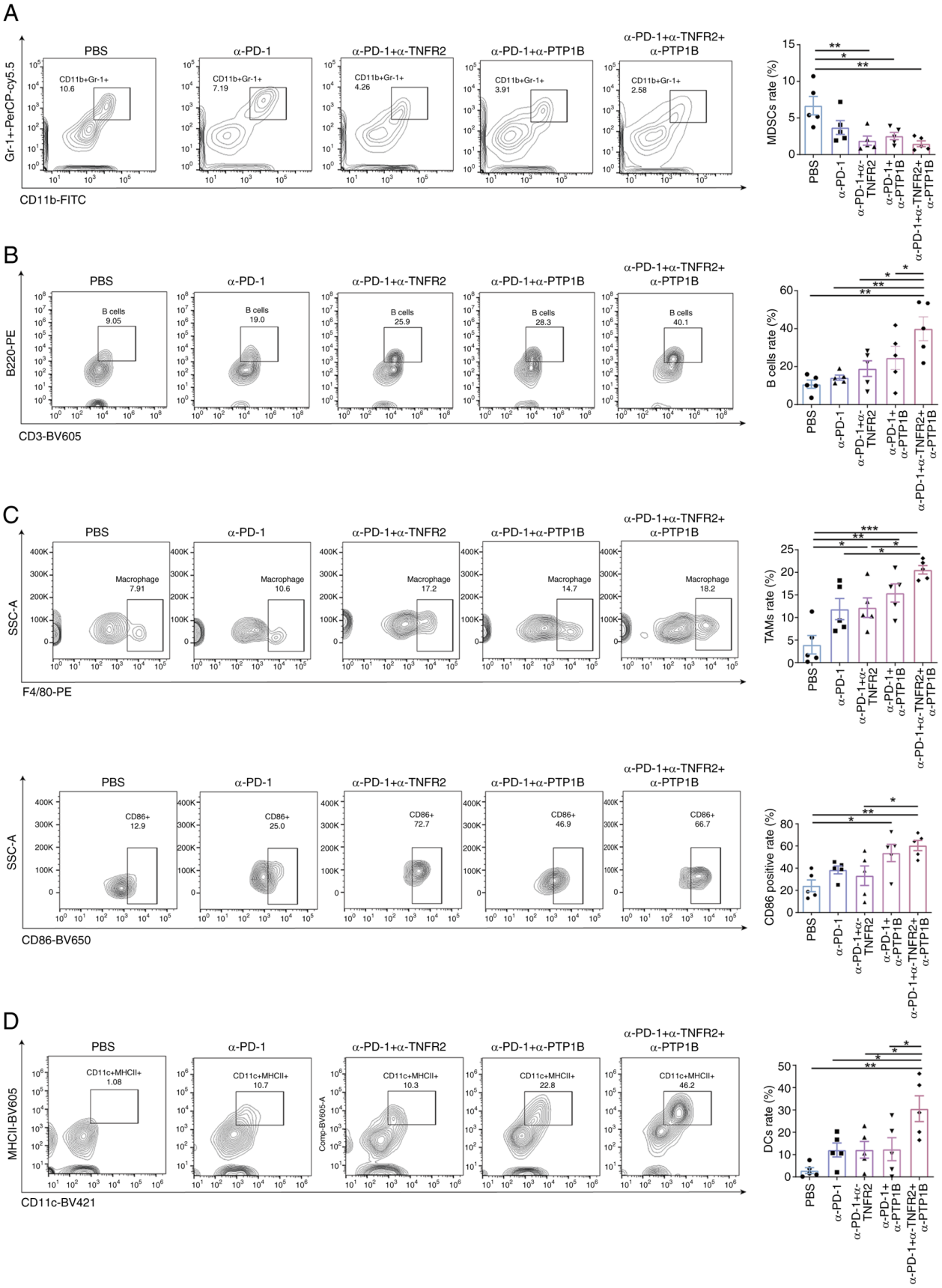

Figure 2. Reconfiguration of immune cell populations within the tumor immune microenvironment of non-small cell lung cancer tumors. (A) The percentages of $CD11b^+$ $Gr\text{-}1^+$ MDSCs in $CD45^+$ cells in tumor (n=5). (B) The percentages of $CD3^+B220^+$ B cells in $CD45^+$ cells in tumor (n=5). (C) The percentages of $F4/80^+$ macrophage cells in $CD45^+CD11b^+$ cells (upper panel) and $CD86^+$ M1 macrophage cells in $F4/80^+$ cells (lower panel) (n=5, per group). (D) The percentages of $CD11c^+MHCII^+$ DCs in $CD45^+$ cells (n=5). The significance of differences in tumor volume was assessed using one-way ANOVA. The survival proportions were compared using the log-rank test. The data were presented as the mean ± SEM. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. MDSCs, myeloid-derived suppressor cells; DCs, dendritic cells; TAMs, tumor-associated macrophages; PTP1B, protein tyrosine phosphatase 1B; PD-1, programmed cell death 1; TNFR2, tumor necrosis factor receptor 2.

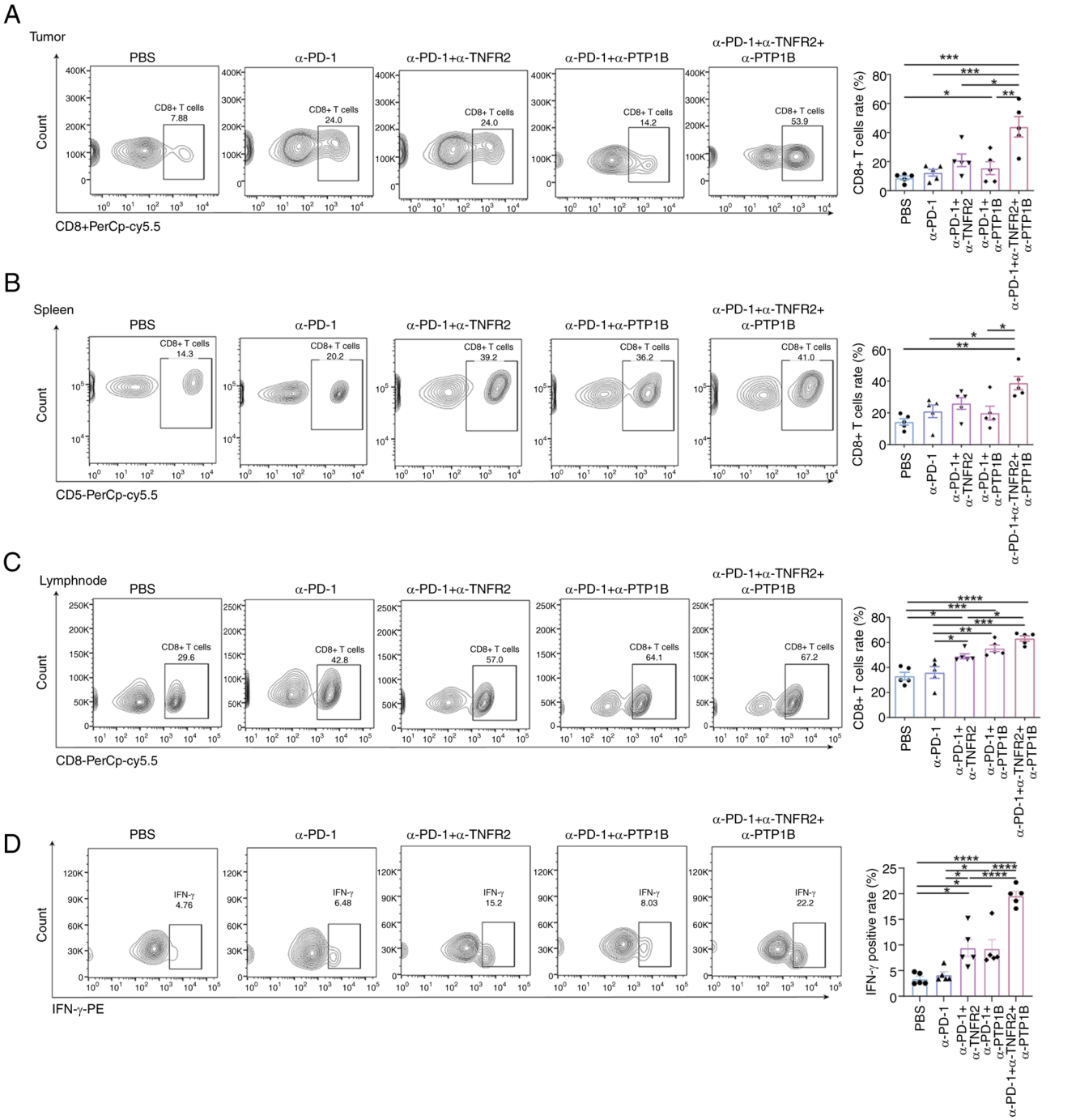

Figure 3. Combination immunotherapy enhances the ratio of CD8⁺T cells and IFN-γ. (A-C) The proportion of cells expressing CD8⁺ in (A) tumors, (B) spleens and (C) lymph nodes, respectively (n=5 per group). (D) The percentage of IFN-γ positive within $CD3^+CD8^+$ cells in mice blood were determined. The statistical significance of differences in tumor volume was evaluated using one-way ANOVA. The data are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. PTP1B, protein tyrosine phosphatase 1B; PD-1, programmed cell death 1; TNFR2, tumor necrosis factor receptor 2.

cells caused by α-PD-1. And the triple therapy triple therapy exhibited a significantly potentiated effect on the immune response of lymph nodes (Fig. 3C). This suggests a localized and systemic recruitment of immune response. Importantly, a significant elevation of IFN-γ was observed in the context of immunotherapy. Specifically, the administration of α-TNFR2 or α-PTP1B significantly enhanced the positive rate of IFN-γ induced by treatment with α-PD-1 in mice. Furthermore, the triple therapy demonstrated a significant amplification in CD8⁺T cells compared with the double treatment groups, thereby enhancing the ratio of IFN-γ on CD8⁺T cells (Fig. 3D). These findings strongly indicated that triple therapy exerts powerful immunomodulatory effects in mice with NSCLC; this combination therapy significantly enhances the recruitment of CTLs, promoting both localized and systemic immunostimulation.

Triple therapy suppresses the immunosuppression of Tregs. TNFR2 dominant expressed on regulatory T cells (Tregs), which are predominantly immunosuppressive (19,20), and

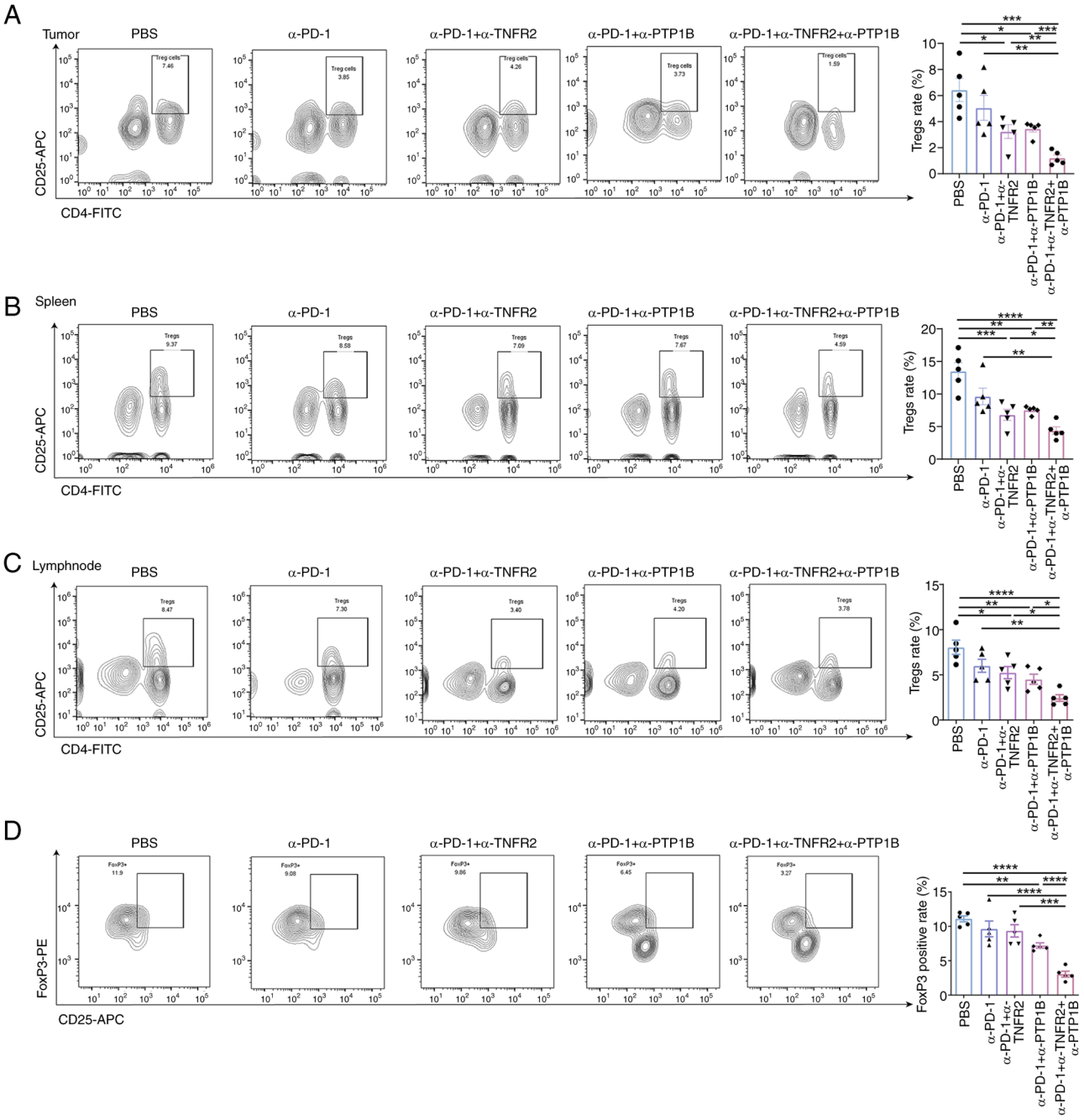

Figure 4. Triple therapy reduces the ratio of Tregs and FoxP3 expression. (A-C) The proportions of $CD4^+CD25^+$ cells in (A) tumors, (B) spleens and (C) lymph nodes were summarized separately, with each group consisting of five samples. (D) The proportion of FoxP3 positive in $CD4^+CD25^+$ cells in mice blood. The significance of differences in tumor volume was assessed using one-way ANOVA. The data are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Tregs, regulatory T cells; PTP1B, protein tyrosine phosphatase 1B; PD-1, programmed cell death 1; TNFR2, tumor necrosis factor receptor 2.

the elevated expression of this factor serves as a promising prognostic indicator in cancers enriched with $CD8^+$T cells. It was recently demonstrated by the authors that the combination of anti-TNFR2 therapy can enhance immune memory against tumors and bolster secondary prevention by suppressing Tregs (21). Another study reported that anti-TNFR2 can induce TAM infiltration while promoting $CD8^+$T cell activation in TIME (19). To investigate the impact of triple therapy on immunosuppression, the distribution of $CD4^+CD25^+$Tregs was analyzed in mouse spleens, lymph nodes and tumors; FoxP3 expression in blood samples was also assessed using flow cytometry. Anti-PD-1 combined with anti-PTP1B or anti-TNFR2 groups demonstrated a reduction in the rate of $CD4^+CD25^+$ Tregs in tumors, spleens and lymph nodes. The triple therapy exhibited the most significant degree of suppression. It was observed that the combination of two or three therapies significantly decreased the proportion of $CD4^+CD25^+$ Tregs. Compared with single and double treatments, the triple therapy evidently diminished the percentage of Tregs in mice tumors (Fig. 4A). Notably, it was found that combination therapies reduced the rate of Tregs in spleens. The triple therapy significantly enhanced the reduction of Tregs (Fig. 4B). In addition, there was a similar response observed in the decrease of Tregs in lymph nodes, as shown

in Fig. 4C. Compared with the PBS group, the combination of anti-PD-1 and anti-PTP1B exhibited a significant reduction in Foxp3 levels in the bloodstream, while the triple therapy demonstrated a significant decrease in the proportion of FoxP3 on $CD4^+CD25^+$ Tregs (Fig. 4D). As expected, the triple therapy had the most pronounced inhibition effect compared with other treatments that included PBS, anti-PD-1 with or without anti-PTP1B or anti-TNFR2. The results demonstrated that the combination immunotherapy effectively suppressed immunosuppression in NSCLC-bearing mice, enhancing effector T cell-mediated antitumor responses.

Triple therapy enhances the expression of PD-L1. The engagement of PD-1 with its ligands, predominantly PD-L1, leads to the attenuation of crucial molecules, resulting in the inhibition of proliferation, activation and cytokine production in immune cells within the TIME. Therefore, to elucidate the local protein expression characteristics within tumors and unravel the pivotal role of anti-PTP1B in combination therapies, the levels of PD-L1, PTP1B and TNFR2 were evaluated in tumor tissues. The results revealed that the combination of anti-PD-1 with either anti-PTP1B or anti-TNFR2 significantly decreases the H-score value of PD-L1 in tumor tissue. Both dual therapy and triple therapy effectively suppressed the expression of PD-L1 (Fig. 5A). Notably, anti-PD-1 combined with anti-PTP1B or anti-TNFR2 reduced the expression of PTP1B, and the triple therapy resulted in a significant reduction compared with other groups (Fig. 5B). The implication here is that the administration of anti-TNFR2 or anti-PTP1B treatments leads to a downregulation of PD-L1 and PTP1B expression, thereby potentially amplifying the immune response through an augmentation in both quantity and functionality of $CD8^+$T cells (Fig. 5C). Moreover, compared with the PBS and anti-PD-1 cohort, the combination of anti-PD-1 with anti-TNFR2 exhibited a significant reduction in TNFR2 expression. The anti-PD-1 + anti-PTP1B group exhibited a significant decrease compared with the triple therapy, indicating that the expression of TNFR2 was reduced by anti-TNFR2 treatment. However, there was no significant effect observed between the anti-PD-1 + anti-TNFR2 group and the triple therapy, suggesting that anti-PTP1B may not influence TNFR2 expression (Fig. 5D). The findings suggested that the collaboration between anti-PTP1B and anti-PD-1 might modulate immune activity by altering PD-L1 expression, while the synergistic modulation of PTP1B and suppression of TIME might be achieved by anti-TNFR2.

Discussion

In the present study, compelling evidence was presented demonstrating that the triple therapy exerts a significant inhibitory effect on tumor growth in a NSCLC model through redistribution of immune cells. Particularly, it predominantly enhanced immunoreactivity and attenuated immunosuppressive effects, which was achieved through a significant increase in $CD8^+$T cells, reduction of Tregs, weakened PD-L1 and TNFR2 within the TIME of NSCLC mice, thus providing evidence in favor of the immunocompetence impact of the triple therapy and suggesting potential therapeutic perspectives.

The reactivation of cytotoxic T cells for the eradication of cancer cells is accomplished through the utilization of antibodies that impede the interaction between PD-1 and PD-L1 (22). The majority of patients with cancer, however, fail to respond to PD-1/PD-L1 blockade due to either elevated or diminished expression of PD-L1, resulting in T-cell exhaustion and enabling tumor cells to elude immune assault. Consequently, this leads to a compromised immune response and unfavorable clinical prognosis for individuals with cancer. Therefore, it is imperative to devise innovative strategies targeting the modulation of immune key molecules' expression and the expansion of immune cells in order to enhance the effectiveness of immunotherapies.

PTP1B, encoded by PTPN1, is a pivotal member of the PTP superfamily, which has been implicated in the pathogenesis of obesity, diabetes, various cancers and cardiovascular diseases (23). PTPN1 was upregulated in hepatocellular carcinoma, and its silencing suppressed proliferation and induced apoptosis of hepatocellular cells (24). The expression of PTP1B is significantly elevated in breast cancer, where it is considered to play a pivotal role in promoting tumorigenesis (25). The present study revealed that PTP1B expression is increased in effector/memory $CD8^+$T cells infiltrating tumors, which is associated with their cytotoxic activity and expansion through JAK/STAT5 signaling coordination (26). Notably, the deletion or inhibition of PTP1B can effectively suppress $CD4^+$T cells, natural killer cells and DCs, while simultaneously promoting the recruitment of Tregs, TAMs, as well as MDSCs. The augmentation significantly enhances the efficacy of CAR-T cells, without triggering autoimmune response or systemic inflammation (15). Therefore, a targeted approach towards PTP1B holds great potential as a promising therapeutic strategy for solid tumors. In the present study, the inhibition of PTP1B significantly decreased the expression of PD-L1 and TNFR2 on lung cancer cell lines, concomitant with the enhancement of $CD8^+$T cells and reducing of $CD4^+$T cells, indicating its pivotal role in modulating both tumor and immune responses in lung cancer. A similar study demonstrated that the inhibition of PTP1B/PTPN2 in T cells enhanced antitumor immunity in immunogenic tumors, while also exerting direct effects on both tumor cells and T cells in cold tumors (27). Due to the presence of PTP1B, the JAK/STAT signaling cascade is closely linked to cytokine-receptor activation in immune cells, which is crucial for the function of activated monocyte-derived DCs (28). The anti-PTP1B may exert a profound influence on the immune system, effectively modulating both cancer cells and their TIME. Through the administration of a synergistic combination of anti-PTP1B and anti-TNFR2, along with PD-1 blockade *in vivo*, there was a remarkable inhibition observed in tumor volume in mice. This treatment regimen resulted in significantly prolonged survival for all the mice involved, without any accompanying side effects. Furthermore, flow cytometry was employed to validate the efficacy of this combination treatment. The triple therapy demonstrated superior efficacy in modulating the immune system distribution in murine models of lung carcinoma, inhibited immune suppressor cells and activated immune effector cells, reorienting the TIME towards an antitumor response. Numerous signaling pathways may be associated with these actions. The GM-CSF-STAT3-TRAF3-PTP1B signaling axis operates

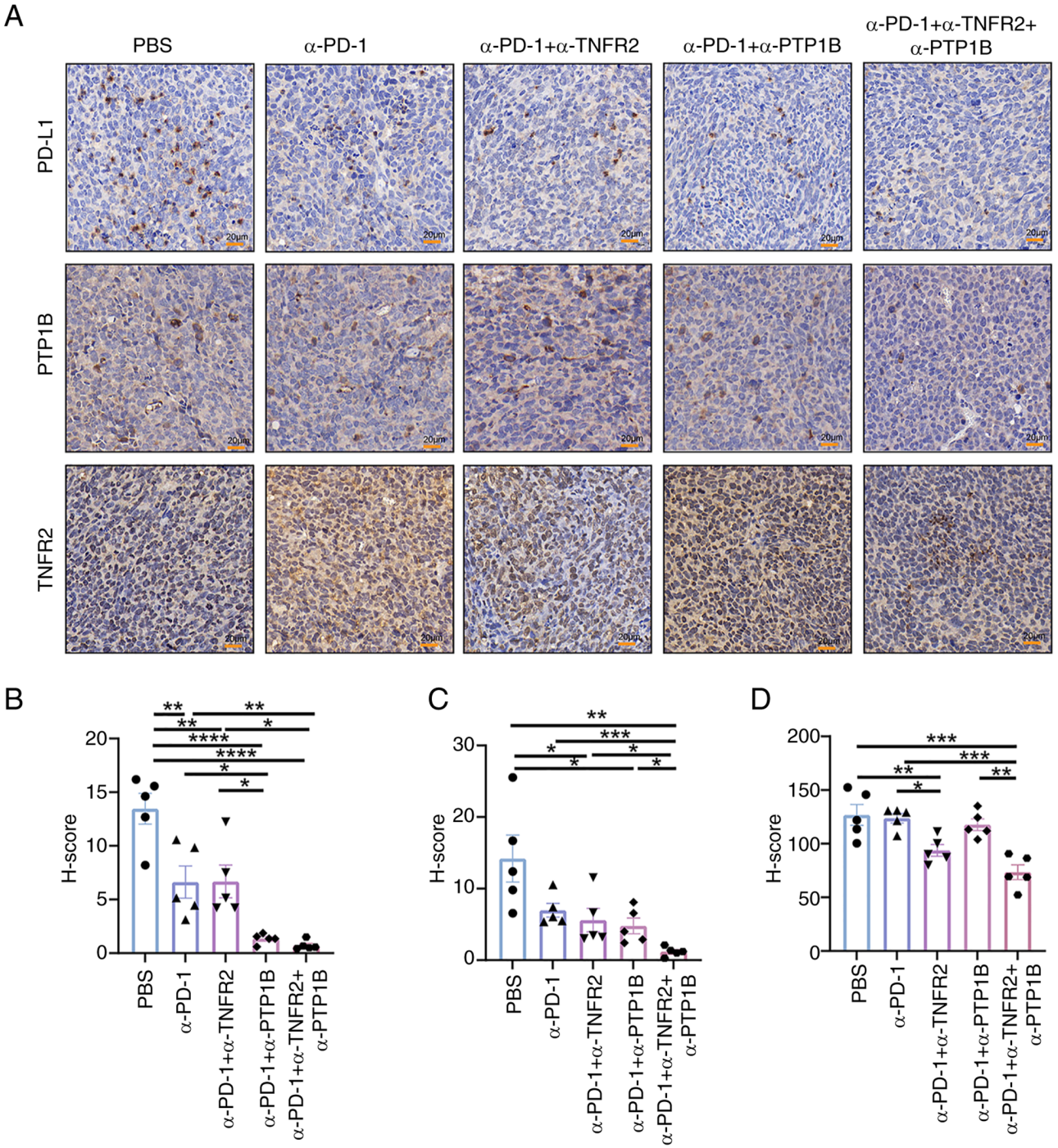

Figure 5. Expression of PD-L1, PTP1B and TNFR2 in non-small cell lung cancer tumors with immunotherapy showcases a resplendent manifestation. (A) The immunohistochemical figures depict the expression of PD-L1, PTP1B and TNFR2. (B) Summary of the H-score of PD-L1 in each group (n=5). (C) Summary of the H-score of PTP1B in each group (n=5). (D) Summary of the H-score of TNFR2 in each group (n=5). The significance of differences was determined using one-way ANOVA. The data are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. PD-L1, programmed cell death-ligand 1; PTP1B, protein tyrosine phosphatase 1B; PD-1, programmed cell death 1; TNFR2, tumor necrosis factor receptor 2.

in inflammatory macrophages and monocytes, collectively regulating the expansion of MDSCs (29). The suppression of M1 cytokines was no longer observed in macrophages derived from PTP1B-KO mice (30). The total ablation of PTP1B can also lead to the hyperactivation of STAT4, STAT1 and Src kinase (28). The expression of PD-L1 in lung cancer cell lines exhibits a positive correlation with MET phosphorylation (31). The PD-L1-associated signaling pathway attenuates macrophage p38α activity by inducing PTP1B (30). The phosphatases suppressor of cytokine signaling 3 (SOCS3), is a crucial gene in tumorigenesis (32); it can synergistically interact with PTP1B to sustain the activation of the JAK-STAT pathway signaling or leptin signaling pathway (33), facilitating immune evasion from $CD8^{+}$T-cell-mediated cytotoxicity (34). The findings present promising prospects for potential therapeutic strategies against cancer.

The triple therapy resulted in an augmentation of $CD8^{+}$T cell proportion and a reduction in Tregs, accompanied with

the enhancement of IFN-γ and alleviation of FoxP3. The findings suggested that the inhibition of PTP1B may confer an enhanced augmenting effect on NSCLC immunotherapy when combined with anti-PD-1 and anti-TNFR2, potentially leading to a more efficacious treatment outcome (17,18). TNFR2, expressed in various tumor cells and Tregs (35), modulates the TGF-β signaling pathway that constitutes a significant proportion within tumors (36), regulating the immune inflammatory signal (37). TNFR2 plays a crucial role in promoting immune evasion, contributing to tumor initiation and progression (21). The combination of PD-1 and TNFR2 synergistically enhances efficacy; however, it results in infiltration of immunotoxin cells within the spleen, indicating suboptimal outcomes. Interestingly, as a pivotal negative regulator for restraining allergic responses, PTP1B-deficient mice exhibited augmented chemotaxis, chemokinesis and trans-endothelial migration in splenic leukocytes (38). Meanwhile, PTP1B is likely to exert inhibitory effects on the vascular endothelial cells through its dephosphorylation of vascular endothelial growth factor receptor 2, a pivotal regulator governing tumor angiogenesis (39,40). Therefore, a triple therapy approach consisting of PD-1 blockade, PTP1B inhibition and anti-TNFR2 immunotherapy was employed to enhance the sensitivity of immunotherapy. A significant amplification in the population of $CD8^+$ T cells was identified, accompanied by a simultaneous reduction in the abundance of Tregs within spleens, lymph nodes and tumors. These findings suggested that triple therapy can modulate the equilibrium of the TIME to favorably enhance immunotherapy sensitivity and elicit systemic immune responses devoid of any associated immune response-related side effects.

Additionally, there are certain limitations to the present study. Since only one cell type was used to explore the immune microenvironment of NSCLC, further investigation of multiple cell types may be more convincing for the efficacy of combination therapy. At the same time, further exploration of the intracellular mechanism of immune cells is of reference significance to clarify the effect of this combination therapy on a variety of tumor cells, which has a guiding role in enhancing the efficacy of immunotherapy for other tumors suitable for immunotherapy. Collectively, the combination of PD-1 blockades and TNFR2 inhibition, with assistance from a PTP1B antibody, activates the immune system and inhibits tumor growth in mice with lung cancer, which may achieve by promoting the immunocompetent cells, and suppressing the immunosuppression. These actions promote survival and prognosis in lung cancer mice. The combination immunotherapy regimen further enhances the efficacy of triple therapy and inhibits lung cancer tumor progression.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HG and HY wrote the original draft. YiN, YuN, HY, QJ, LL, HL, QW, LZ, SCh and MW wrote, reviewed and edited the manuscript. YiN, YuN, HY and XZ performed the conceptualization. HY, XZ, QJ and LL performed the data analysis. QW, LZ, SCh, LL and MW performed the formal analysis. YiN, YuN, HY, QJ, HL, QW, LZ, SCh, MW and XZ designed the methodology, created the animal models and performed treatment experiments. QJ, LL, QW and MW performed the project administration. LZ, SCh and HG provided study materials and reagents. YiN and XZ performed the supervision. YiN and HY performed the validation. HY and YuN generated the figures. XZ, YiN and HG provided the funding. HG, HY and YiN confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments involving animals were conducted according to the ethical policies and procedures approved (approval no. 2305196) by the Animal Research Ethics Committee of Guizhou Medical University (Guiyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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