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Single-Cell Sequencing Exploration of Extrinsic Adaptive Resistance to PD-1 Inhibitors: Theory of Acupuncture-Associated Intervention in Advanced Non-Small Cell Lung Cancer

(Penerokaan Penjujukan Sel Tunggal Rintangan Adaptif Ekstrinsik terhadap Perencat PD-1: Teori Intervensi Berkaitan Akupunktur dalam Kanser Paru-paru Bukan Sel Kecil Tahap Teruk)

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ABSTRACT

PD-1 and PD-L1 are respectively the receptor-ligand molecules on T cells and their target cells. The interaction between them can mediate immunosuppressive signals and induce immune escape of tumors. PD-1/PD-L1 inhibitors have revolutionized advanced non-small cell lung cancer (NSCLC) treatment, both primary resistance and acquired resistance persist due to the immunosuppressive tumor microenvironment and alternative immune checkpoints. Acupuncture shows immunomodulatory potential, but its synergy with PD-1 blockade remains unclear. To investigate the underlying mechanisms, we examined single-cell RNA sequencing profiles derived from NSCLC patients prior to and following PD-1 inhibitor treatment, utilizing the GSE207422 dataset. After quality control, 53,360 cells were retained. Cell types were annotated via unsupervised clustering, all cell types and T cell subsets were clustered, and T cells differentiation trajectories were reconstructed. Intercellular communication was assessed with CellChat, and a mechanistic framework for acupuncture's synergistic effects was integrated from the literature. PD-1 inhibitor treatment remodeled the tumor microenvironment: T cells increased from 23.9% to 49.0%, while tumor cells decreased. CD8+ cytotoxic T cells expanded from 32.4% to 55.7% of T cells, whereas CD4+ naïve T cells declined from 67.1% to 42.7%. Pseudotime analysis indicated differentiation from CD4+ naïve toward CD8+ cytotoxic and CD8+ memory T cells. CellChat showed extensive T cell–myeloid interactions. Notably CD99-CD99 was highly expressed between T cells and tumor cells, suggesting an adaptive resistance mechanism. PD-1 inhibitors drive potent anti-tumor immunity in NSCLC, marked by CD8+ T cells expansion and tumor cells reduction, but tumor cells concurrently upregulate CD99-mediated compensatory suppression. We propose that acupuncture may synergize by enhancing T cell functional fitness, enabling T cells to resist CD99-PILRα inhibitory signaling, offering a promising strategy to overcome immunotherapy resistance.

Keywords: Acupuncture; immune checkpoint; non-small cell lung cancer; PD-1 inhibitor; single-cell RNA sequencing

ABSTRAK

PD-1 dan PD-L1 masing-masing merupakan molekul reseptor-ligan pada sel T dan sel sasarannya. Interaksi antara mereka boleh menjadi perantara isyarat imunosupresif dan mendorong pelepasan imun tumor. Perencat PD-1/PD-L1 telah merevolusikan rawatan kanser paru-paru bukan sel kecil (NSCLC) tahap teruk, kedua-dua rintangan primer dan rintangan yang diperoleh berterusan disebabkan oleh persekitaran mikro tumor imunosupresif dan titik semak imun alternatif. Akupunktur menunjukkan potensi imunomodulatori, tetapi sinerginya dengan sekatan PD-1 masih tidak jelas. Untuk mengkaji mekanisme yang mendasari, kami memeriksa profil penjujukan RNA sel tunggal yang diperoleh daripada pesakit NSCLC sebelum dan selepas rawatan perencat PD-1, menggunakan set data GSE207422. Selepas kawalan kualiti, 53,360 sel dikekalkan. Jenis sel telah dianotasi melalui pengelompokan tanpa pengawasan, semua jenis sel dan subset sel T telah dikelompokkan dan trajektori pembezaan sel T telah dibina semula. Komunikasi antara sel telah dinilai dengan CellChat dan rangka kerja mekanistik untuk kesan sinergi akupunktur telah disepadukan daripada kepustakaan. Rawatan perencat PD-1 mengubah suai persekitaran mikro tumor: Sel T meningkat daripada 23.9% kepada 49.0%, manakala sel tumor menurun. Sel T sitotoksik CD8+ berkembang daripada 32.4% kepada 55.7% sel T, manakala sel T naif CD4+ menurun daripada 67.1% kepada 42.7%. Analisis pseudomasa menunjukkan pembezaan daripada CD4+ naif terhadap sel T memori sitotoksik CD8+ dan CD8+. CellChat mendedahkan interaksi sel T-myeloid yang meluas. CD99-CD99 diekspresikan dengan tinggi antara sel T dan sel tumor, menunjukkan mekanisme rintangan adaptif. Perencat PD-1 memacu imuniti anti-tumor yang kuat dalam NSCLC, ditandai dengan pengembangan sel T CD8+ dan pengurangan sel tumor, tetapi sel tumor secara serentak meningkatkan penindasan pampasan yang

dimediasi CD99. Kami mencadangkan bahawa akupunktur boleh bersinergi dengan meningkatkan kecergasan fungsi sel T, membolehkan sel T menentang isyarat perencat CD99-PILRα, memberikan strategi yang berpotensi untuk mengatasi rintangan imunoterapi.

Kata kunci: Akupunktur; kanser paru-paru bukan sel kecil; penjujukan RNA sel tunggal; perencat PD-1; pusat pemeriksaan imun

INTRODUCTION

Globally, lung cancer is the primary cause of cancer-related mortality. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of cases, with those diagnosed at advanced stages generally facing a poor prognosis (Bray et al. 2024; Sung et al. 2021). Programmed Death Receptor 1 (PD-1) and Programmed Death Ligand 1 (PD-L1) are immune checkpoint molecules that regulate the immune response by inhibiting the activity of T cells. With the advent of immune checkpoint inhibitors targeting the PD-1/PD-L1 axis, the standard of care for advanced NSCLC has shifted dramatically, offering remission and extended survival for a subset of patients (Gandhi et al. 2018; Garon et al. 2019). Nevertheless, the overall response rate with PD-1/PD-L1 monotherapy still falls below 30%, and both primary and acquired resistance present considerable hurdles in clinical practice (Morad et al. 2021; O'Donnell, Teng & Smyth 2019). This resistance frequently arises from immunosuppressive characteristics within the tumor microenvironment, along with elevated expression of alternative immune checkpoints—both of which collectively impair T cell effector function (Pitt et al. 2016; Sharma et al. 2017).

In this context, identifying and targeting novel immune checkpoints has emerged as a promising strategy for overcoming drug resistance and enhancing the efficacy of immune checkpoint inhibitors (Kraehenbuehl et al. 2022; Tang et al. 2018). A pioneering study previously published identified the paired immunoglobulin-like type 2 receptor alpha (PILRα) as a key immune checkpoint. This study demonstrated that PILRα expressed on tumor cells can directly interact with the CD99 molecule on T cells. Via this interaction, key signaling cascades—including ZAP70, NFAT, IL-2, JAK, and STAT—are blocked, resulting in impaired T cell activation, proliferation, and cytotoxic function (Xia et al. 2025). Notably, the high expression of PILRα in various human cancers is associated with poor patient prognosis, and preclinical models have shown that blocking the PILRα-CD99 axis can synergize with anti-PD-1 therapy to jointly inhibit tumor growth (Dong et al. 2002; Xia et al. 2025). This discovery positions the PILRα-CD99 axis as a highly attractive new therapeutic target (Chen & Han 2015; Taube et al. 2012).

At the same time, there is an increasing number of studies integrating supplementary therapies to enhance the efficacy of immunotherapy (Liu et al. 2024; Wang et al. 2023). Acupuncture, as a key component of traditional Chinese medicine, has gradually gained recognition in the scientific community for its immunomodulatory properties (Hui et al. 2000; Johnston et al. 2011). According to clinical studies, electro-acupuncture boosts T-cell-mediated immunity by enhancing CD4+ and CD8+ T cell expansion, elevating the secretion of effector cytokines such as interferon− γ and interleukin−2 (IL-2), and modulating the Th1/Th2 balance (Chen et al. 2017; Xu et al. 2025). Moreover, new evidence suggests that acupuncture can reshape the immunosuppressive tumor microenvironment (Wang et al. 2024). A recent retrospective study, which included 217 patients with NSCLC treated with immune checkpoint inhibitors, found that the median progression-free survival of patients receiving acupuncture treatment was significantly prolonged, especially in the bone metastasis subgroup, where the benefits were more pronounced (Zhou et al. 2025). Therefore, it remains unclear whether acupuncture can improve outcomes related to immunotherapy resistance in cancer patients. Based on available literature, we reasonably speculate that acupuncture may improve the prognosis of cancer patients receiving immunotherapy, which is expected to provide novel strategies for clinical practice.

In this study, we utilized single-cell RNA sequencing(scRNA-seq) technology to investigate the dynamic changes in the tumor microenvironment of advanced NSCLC patients treated with PD-1 inhibitors, and proposed a hypothesis: Acupuncture can enhance the functional adaptability of T cells, enabling them to resist the inhibitory signals mediated by the PILRα-CD99 axis, thereby fully releasing the potential of PD-1 blockade, driving a powerful tumor clearance effect, improving the prognosis of advanced NSCLC patients, and benefiting more advanced NSCLC patients.

METHODS

DATA SOURCE

This study is a retrospective bioinformatics analysis based on publicly available dataset. The scRNA-seq dataset was downloaded from the GEO repository under accession number GSE207422.

PROCESSING OF scRNA-seq DATA

The Seurat software package was used to preprocess the raw gene expression matrix (Stuart et al. 2019). High-quality cells were obtained based on the following

criteria: removing cells with extremely high or low values for nFeature and nCount, and mitochondrial gene expression in a single cell being less than 20% of the total, and erythrocyte gene expression in a single cell being less than 5% of the total.

The DoubletFinder package was used to remove cell bimodality. After sample normalization, the top 2,000 variable genes were selected and scaled. To correct for batch effects across samples, PCA was performed using the Harmony method, and the first 30 principal components were retained for uniform manifold approximation and projection (UMAP) dimensionality reduction and subsequent visualization of gene expression. Cell clusters were annotated based on known markers from the literature and the CellMarker database (http://xteam.xbio.top/CellMarker/). We then examined the relative proportions of the identified cell types.

DIFFERENTIALLY EXPRESSED GENES (DEGs) AND ENRICHMENT ANALYSIS

Using the Seurat package, we applied the FindAllMarkers function to identify DEGs for each cell type based on the standardized expression data (Butler et al. 2018). Candidate genes were required to be expressed in more than 25% of cells within a given cluster and to have a $\log_2$ fold change (logFC) greater than 0.25. For Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, statistical significance was set at an adjusted P-value of less than 0.05. Enrichment of cluster−specific biomarker genes was performed with the ClusterProfiler package (Yu et al. 2012).

We utilized the FindAllMarkers function (Wilcoxon rank-sum test) to identify differential genes specific to T cell subgroups, and conducted GO and KEGG enrichment analyses with clusterProfiler to explore heterogeneity within these subsets.

DETERMINATION OF CELL SUBPOPULATIONS

Extract all T cells, re-perform normalization processing, identify the top 2,000 variable genes, and standardize their data. Subsequently, we conduct a PCA analysis on them, using the harmony method to eliminate batch effects among samples. Cell types were assigned by evaluating the expression patterns of established marker genes within each cluster. Clusters exhibiting similar expression profiles were grouped together as the same cell type and visualized using UMAP for two−dimensional projection.

TRAJECTORY ANALYSIS

Firstly, we further utilized the Monocle software toolkit to reconstruct the cell differentiation trajectory. We employed DDRTree for dimensionality reduction and observed the developmental process of subpopulation cells on the newly established trajectory. Finally, we used the slingshot method to further analyze the cell trajectory during T cell differentiation, using it to infer the cell lineage.

ANALYSIS OF INTERCELLULAR INTERACTIONS

To investigate intercellular communication between T cell subsets and other cells within the tumor microenvironment, we applied the CellChat package to analyze ligand−receptor interactions between these populations (Vento-Tormo et al. 2018). This approach enabled us to infer cell−cell communication at both the signaling pathway and receptor−ligand levels, as well as to examine how distinct signaling pathways coordinate across different cell types.

RESULTS

PROFILING VIA scRNA-seq ENABLED IDENTIFICATION OF THE MAJOR CELLULAR POPULATIONS PRESENT IN NSCLC PATIENTS PRIOR TO AND FOLLOWING PD-1 INHIBITOR THERAPY

To verify the effectiveness of PD-1 inhibitor treatment in NSCLC patients, we collected samples from patients with stage IIIA NSCLC in the GSE207422 dataset for scRNA-seq, obtaining the main cell types of NSCLC before and after PD-1 inhibitor treatment. To explore its efficacy, we mainly evaluated the changes in immune cells (mainly T cells) before and after treatment.

Following initial quality control and batch effect correction, 53,360 cells remained across both pre− and post−treatment samples. Dimensionality reduction clustering of these cells identified 15 distinct clusters (Figure 1(A)). Based on tissue−specific canonical markers reported in the literature, the clusters were classified into 13 major cell types: Proliferating Cell (1754), T cell (25,460), NK Cell (1,350), Tumor Cell (2,878), Macrophage (5,561), Monocyte (3,877), Plasma Cell (2,054), Neutrophil (3,972), B Cell (5,295), Cancer-Associated Fibroblast (239), Mast Cell (340), Cililated Epithelial Cell (158), and Alveolar Type II Cell (422) (Figure 1(B)). When the cells before and after treatment were displayed separately, it was found that the types of cells were exactly the same before and after treatment, but the proportions of each cell type varied greatly before and after treatment (Figure 1(C)). Compared with before treatment, the proportion of T cells increased significantly, from 23.9% to 49%, while the proportion of Tumor Cells decreased significantly (Figure 1(D)-1(E)). This indicates that after treatment with the PD-1 inhibitor, the patients' immune function has been enhanced, and tumor proliferation has been effectively inhibited.

The expression levels of marker genes for the 13 cell types are not the same. Each marker gene in

each cell type was displayed through a bubble chart (Figure 1(F)). Volcano plots illustrated DEGs for each of the 13 cell types, comparing pre− and post−treatment samples (Figure 1(G)). A heatmap was used to show the results of GO enrichment analysis and KEGG enrichment analysis of the DEGs between cell types before and after treatment (Figure 1(H)-1(I)). It could be seen that before and after treatment, the biological processes in immune cells T Cells, which we most concerned about are mainly cell differentiation, including: Lymphocyte differentiation, T cell differentiation, Mononuclear cell differentiation. The biological processes corresponding to Tumor Cells include: Aerobic respiration, Cellular respiration and Oxidative phosphorylation. Meanwhile, the KEGG pathways corresponding to T Cells were mainly T cell receptor signaling pathway, Th1 and Th2 cell differentiation, Th17 cell differentiation.

VISUALIZATION OF T CELL SUBPOPULATIONS

The most crucial cellular change in immunotherapy is that CD8+ T cells are 'awakened' from the exhausted state and undergo significant expansion to become CD8+ Cytotoxic T Cells (Jiang et al. 2025; Yousif et al. 2025). Next, we used scRNA-seq data from T cells to conduct further sub-clustering, resulting in three cell subpopulations and labeling their cell counts: CD4+ Naive T Cell (10,930), CD8+ Cytotoxic T Cell (13,877), CD8+ Memory T Cell (381) (Figure 2(A)). By comparing the number and proportion of T cell subsets before and after treatment, it was found that the number of cells in each T cell subset increased after treatment (Figure 2(B)). Additionally, it was observed that the proportion of CD8+ Cytotoxic T Cells after treatment significantly expanded compared to before treatment, increasing from 32.4% before treatment to 55.7% after treatment. This indicated that the exhausted T cells were reactivated and regained their proliferative capacity (Cillo et al. 2024). At the same time, the proportion of CD4+ Naive T Cells after treatment significantly decreased compared to before treatment, dropping from 67.1% to 42.7% (Figure 2(C)). This may be due to the mobilization of peripheral immune reserves, where the initial cells are activated and differentiate into effector or memory cells, which is also the reason for the increase in the proportion of CD8+ Memory T Cells after treatment compared to before treatment (Edwards et al. 2025).

Volcano plots were used to depict the DEGs before and after treatment for these three T cell subpopulations (Figure 2(D)). Figure 2(E)-2(F) presented heatmaps illustrating the GO enrichment analysis outcomes for T cells and their subpopulations, comparing pre- and post-treatment differences. It can be observed that before and after treatment, the biological processes corresponding to CD4+ Naive T Cell mainly included Positive regulation of lymphocyte activation. The biological processes corresponding to CD8+ Cytotoxic T Cell mainly included MHC class II protein complex assembly and Peptide antigen assembly with MHC class II protein complex. The biological processes corresponding to CD8+ Memory T Cell mainly included Leukocyte mediated immunity. In KEGG enrichment analysis, the enriched pathways corresponding to CD4+ Naive T Cell mainly included Cytokine−cytokine receptor interaction and Th17 cell differentiation. The enriched pathways corresponding to CD8+ Cytotoxic T Cell mainly included Graft−versus−host disease and Allograft rejection (Figure 2(G)).

VISUALIZATION OF PSEUDOTIME ANALYSIS OF T CELLS USING SLINGSHOT AND MONOCLE

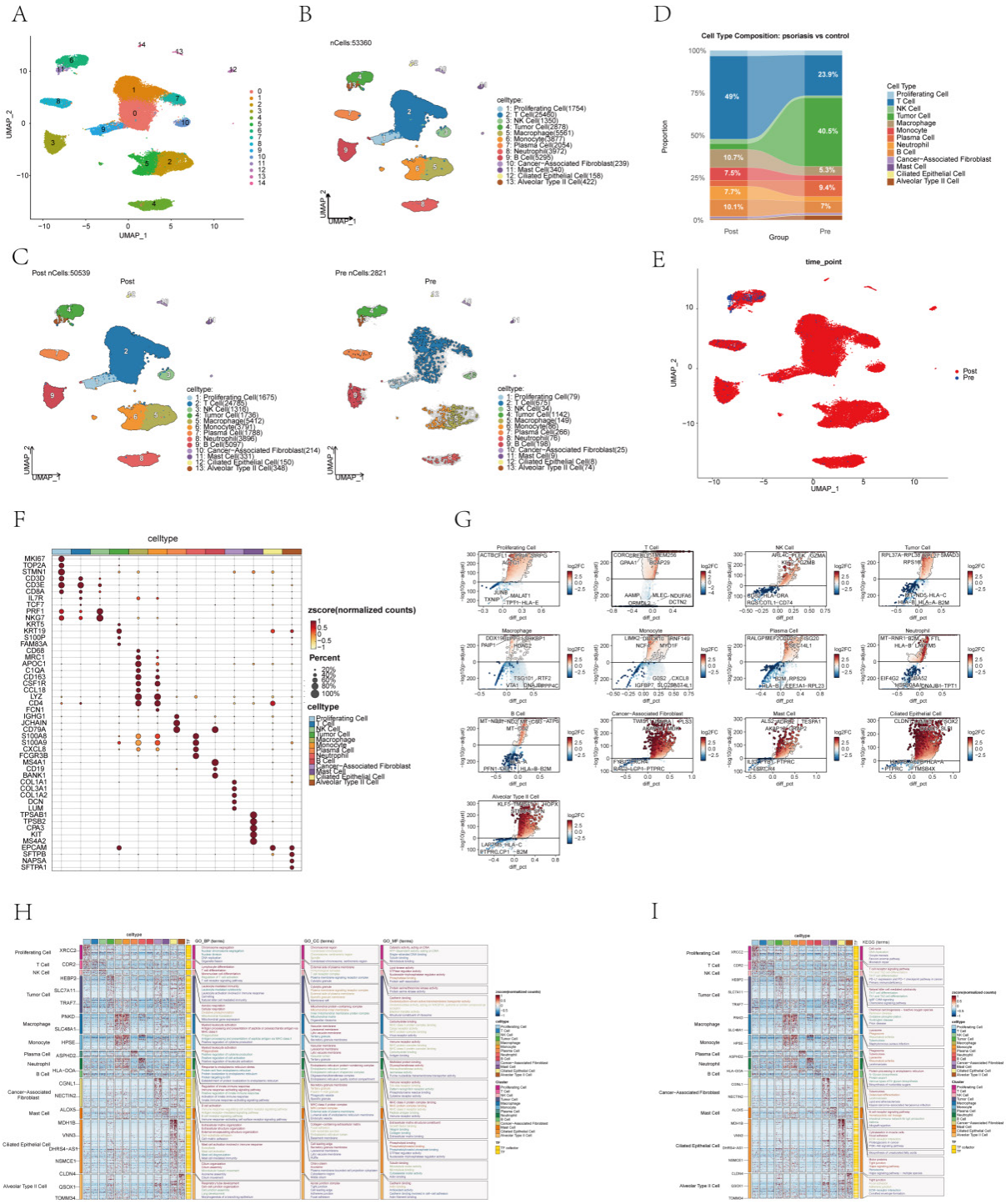
To investigate the differentiation trajectories among the three T cell subpopulations, we applied slingshot for lineage inference and visualized the results. It can be observed that before and after the treatment, the T cell subpopulations underwent differentiation from CD4+ naive T cells to CD8+ cytotoxic T cells and CD8+ memory T cells (Figure 3(A)).

A pseudotime analysis of T cell changes during the treatment process was conducted to investigate the differentiation process of T cells in NSCLC patients treated with PD-1 inhibitors. The UMAP plot was used to show the distribution of the pseudotime of cell subpopulations, and it can be observed that the T cell subpopulations show continuous differentiation in the time series, and generally present a trend of differentiation from left to right (Figure 3(B)).

To investigate the origin of T cell subpopulations, a pseudotime trajectory analysis was further conducted on three T cell subpopulations. The CD4+ Naive T Cell subpopulation was mainly observed at the beginning of the pseudotime trajectory and in the trajectories to the right of the bifurcation point. The distribution of the CD8+ Cytotoxic T Cell subpopulation was similar to that of the CD4+ Naive T Cell subpopulation, but it was mainly concentrated in the trajectories to the left of the bifurcation point. The CD8+ Memory T Cell subpopulation was mainly concentrated in the trajectories to the left of the bifurcation point (Figure 3(C)-3(D)).

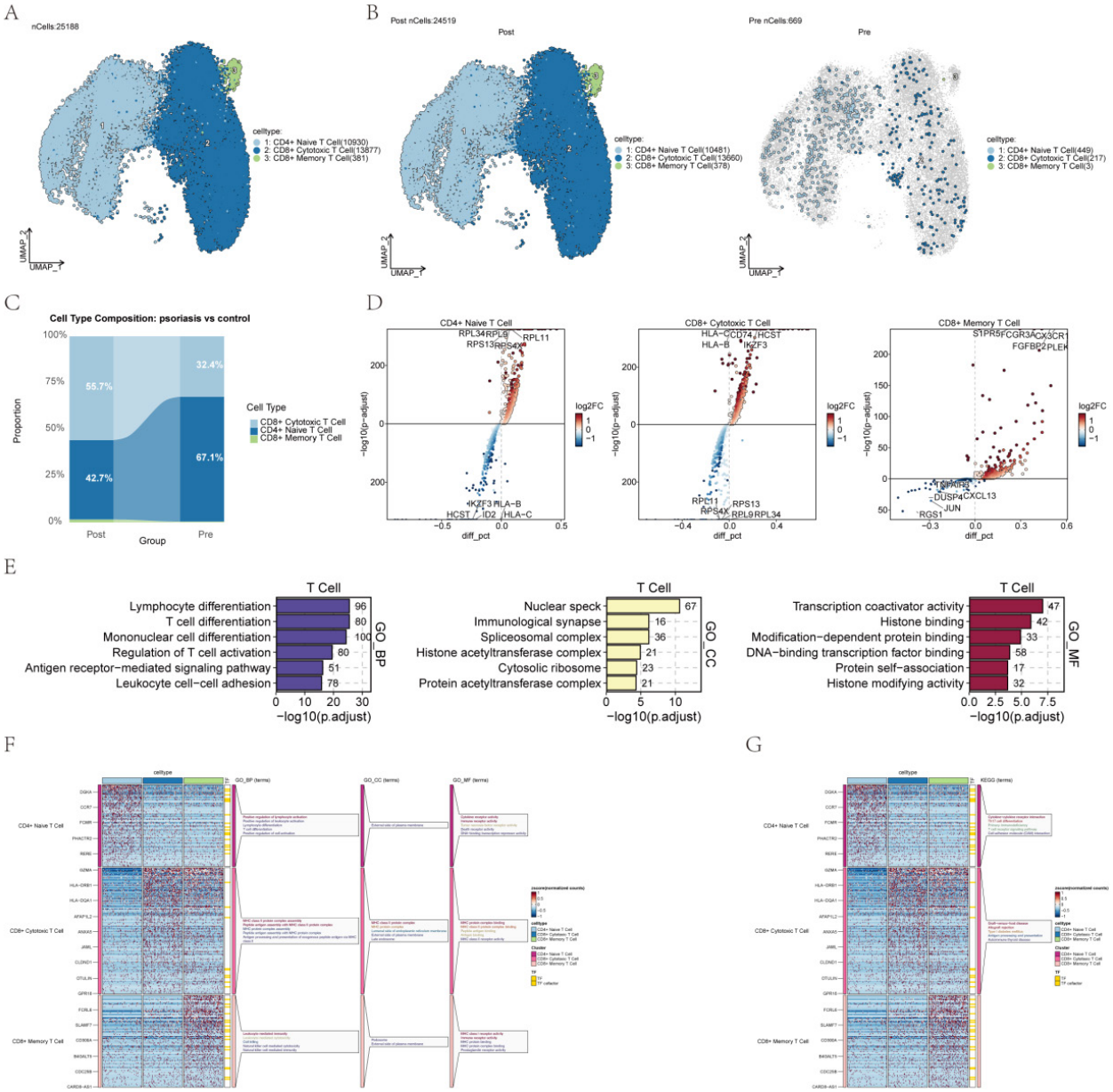
ANALYSIS OF CELL-TO-CELL COMMUNICATION

To systematically dissect the complex cellular interactions, we mapped both cell−cell relationships and ligand−receptor communication networks. Using CellChat, we first constructed intercellular communication networks spanning major cell populations, including T cells, macrophages, and NK cells. The number of interactions was calculated (represented by the 'lines' connecting the two cell types, the thicker the line, the more interaction pathways) (Figure 4(A)). When the cell communication network of T Cells alone was displayed,



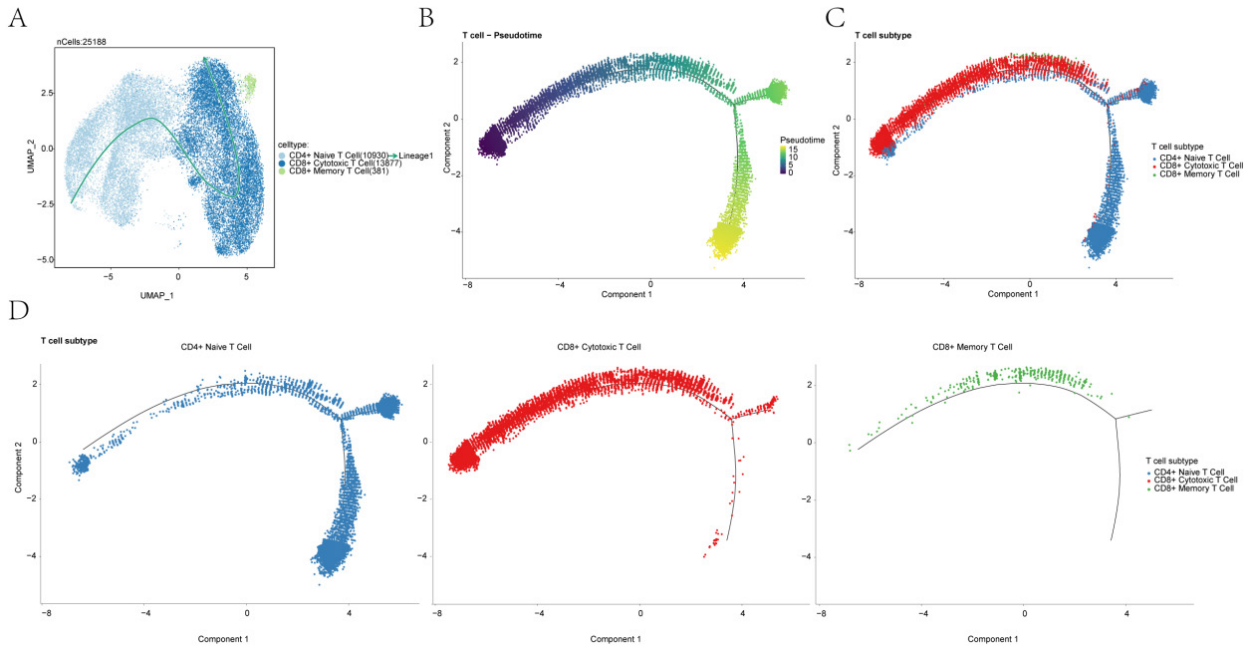
A: UMAP visualization of 15 distinct cell clusters from all NSCLC samples before and after treatment, with each cell colored according to its corresponding cluster. B: UMAP projection displayed 13 cell types annotated from the 15 cell clusters in NSCLC samples before and after treatment, with distinct colors indicating different cell lineages. C: UMAP comparison of cellular composition and abundance in NSCLC patients before and after treatment; the right panel represented the pre-treatment status, while the left panel showed the post-treatment profile. D: Percentage stacked bar chart illustrated shifts in the relative proportions of each cell type before and after treatment in NSCLC patients. E: UMAP visualization of overall cellular distribution in NSCLC samples before and following treatment. F: Dot plot showed the differential gene expression profiles across cell types before and after treatment. Dot size indicated the proportion of cells expressing the gene within each cell type, and color intensity reflected the relative gene expression level. G: Volcano plots showed the distribution of differentially expressed genes in the 13 identified cell types between pre- and post-treatment conditions. H: GO enrichment analysis showed the biological processes, cellular components, and molecular functions associated with the 13 cell types before and after treatment. I: KEGG enrichment analysis identified the key signaling pathways related to the 13 cell types in NSCLC patients before and following PD-1 inhibitor therapy.

FIGURE 1. Single-cell RNA sequencing identified the major cell populations in advanced NSCLC patients before and after PD-1 inhibitor treatment



A: UMAP projection depicted three T cell subpopulations in NSCLC patients before and after treatment, along with the corresponding cell count for each subpopulation. Each dot represented a single cell, colored on the basis of its T cell subpopulation identity. B: UMAP illustrations compared the cellular distribution and abundance of the three T cell subsets between pre- and post-PD-1 inhibitor treatment. Individual cells were colored according to their respective subsets. C: Percentage stacked bar chart displayed alterations in the relative proportions of the three T cell subsets in NSCLC patients before and following treatment. D: Volcano plots illustrated the expression profiles of differentially expressed genes within the three T cell subsets. E: GO enrichment analysis showed the biological processes, cellular components, and molecular functions associated with total T cells in NSCLC patients before and after treatment. F: GO enrichment analysis identified the biological processes, cellular components, and molecular functions linked to each of the three distinct T cell subsets before and following treatment. G: KEGG enrichment analysis uncovered the key signaling pathways associated with the three T cell subsets in NSCLC patients before and after PD-1 inhibitor therapy.

FIGURE 2. Visualization of T cell subpopulations



A: UMAP projection showed the differentiation trajectory of T cells exhibiting a temporally ordered pattern. B-D: UMAP visualizations illustrated the pseudotime distribution of the three T cell subpopulations, respectively.

FIGURE 3. Visualization of T cell pseudotime trajectory analysis using Slingshot and Monocle

it was found that the number of interactions between T Cells and Monocytes and Macrophages was relatively large (Figure 4(B)). The ligand-receptor pairs between T Cells and other cells showed that T Cells mainly showed high expression of the CD99-CD99 ligand-receptor pair with Tumor Cells (Figure 4(C)). CD99 is a key molecule for forming an immunological synapse on T Cells. Studies have shown that CD99 on T Cells interacts with actin and microtubules through its transmembrane region and intracellular region, which is crucial for stabilizing the immune synapse between T Cells and target cells (Manara et al. 2025). Therefore, this interaction may imply that T Cells are actively binding to tumor cells and attempting to exert killing functions. The CD99-related pathway mainly contributes to extrinsic adaptive resistance after PD-1 inhibitor treatment, rather than intrinsic primary resistance inherent to tumor cells (Xia et al. 2025). This refined classification helps precisely distinguish the immunoresistance mechanism mediated by intercellular communication in the tumor microenvironment.

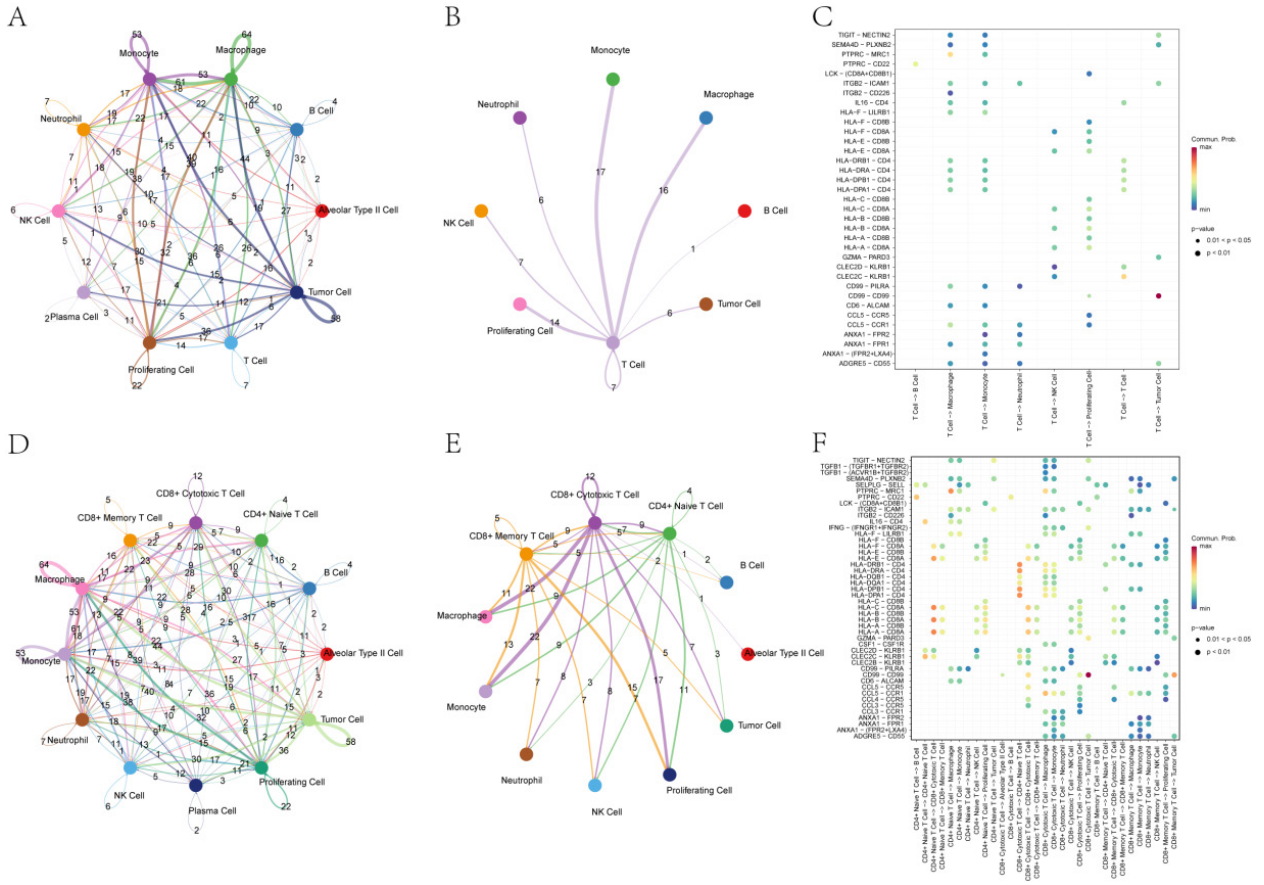
When the cell communication networks between T cell subpopulations and other various cells are separately presented, it can be observed that the number of interactions between CD8+ Cytotoxic T Cells and Monocytes and Macrophages is relatively large, similar to the overall results of T Cells. This may be related to the relatively high proportion of CD8+ Cytotoxic T Cells in T cells after treatment (Figure 4(D)-4(E)). By presenting the ligand-receptor pairs of interactions between T cell subsets and various cell types, it can be found that the CD99-CD99 ligand-receptor pair is mainly highly expressed between CD8+ Cytotoxic T Cells and Tumor Cells (Figure 4(F)).

EXPLORING THE POSSIBILITY OF ENHANCING THE THERAPEUTIC EFFECT OF ADVANCED NSCLC PATIENTS THROUGH THE COMBINATION OF ACUPUNCTURE AND PD-1 INHIBITORS

In this study, single-cell sequencing showed three key pieces of information: the proportion of T cells (especially CD8+ Cytotoxic T Cells) significantly increased after treatment, the proportion of tumor cells decreased significantly, and the CD99-CD99 ligand-receptor pairs between T cells and tumor cells were highly expressed.

The doubling of T cell proportion and the decrease in tumor proportion jointly constituted the evidence for the efficacy of PD-1 inhibitor treatment in NSCLC patients: the immune system has been successfully activated, and the activated T cells effectively cleared the tumor cells (Xu et al. 2025). However, the high expression of CD99-CD99 provided us with a deeper perspective.

Combined with the pioneering studies previously published, this phenomenon is likely to represent that the tumor cells are initiating a ‘countermechanism’ - by binding to the PILR α on the surface and CD99 on the T cells, forming a new immune checkpoint axis, attempting to inhibit the function of T cells (Xia et al. 2025). This suggested that behind the strong therapeutic



A: Circular plot displayed the number of intercellular interactions among all cell types. B: Circular plot illustrated the quantity of interactions among T cells and the other 12 cell types. C: Cell communication interaction plot showed the intensity of ligand-receptor interactions among T cells and other cell populations. D: Circular plot presented the number of interactions among T cell subpopulations and all other cell types. E: Circular plot depicted the quantity of interactions among T cell subpopulations and the other 12 cell types. F: Cell communication interaction diagram illustrated the intensity of ligand-receptor interactions among the three T cell subpopulations and other cell types.

FIGURE 4. CellChat analysis of intercellular communication across all cell types

effect we observe, the tumor cells are attempting to 'apply the brakes'. Meanwhile, how does acupuncture help T cells overcome these obstacles? We believed that this might be the key mechanism by which acupuncture works in conjunction with PD-1 inhibitors to improve the prognosis of NSCLC.

Firstly, acupuncture had been proven to systematically enhance the function of T cells. Earlier research demonstrated that combining acupuncture with PD-1 inhibitors can greatly boost the count of CD4+ and CD8+ T cells, with RNA-Seq highlighting the activation pathways of T cells (Xu et al. 2025). Electro-acupuncture at Zusanli (ST36) significantly elevates serum IFN-γ and IL-2 levels and enhances CD4+ T cell expression via the TRPV channel (Chen et al. 2017).

Secondly, the functionally enhanced T cells could better resist immunosuppression. A review highlighted that acupuncture can modify the immune microenvironment by improving CD8+ T cell function and reestablishing the Th1/Th2 equilibrium (Liu et al. 2024). This 'immune normalization' effect enables T cells to have stronger resistance when facing inhibitory signals from tumor cells through the PILRα-CD99 axis (Xia et al. 2025).

Therefore, we proposed a core hypothesis: Acupuncture could enhance the activation and effector functions of T cells, reshape their metabolic adaptability and signal transduction capabilities, enable them to resist immune suppression mediated by the PILRα-CD99 axis. The functionally enhanced T cells are no longer easily 'braked', allowing PD-1 inhibitors to exert their role of relieving PD-1 inhibition more fully.

The supportive effect of acupuncture enabled T cells to overcome the countermeasures of tumors and ultimately achieve a significant clearance of tumor cells. The previously published Meta-analysis supported this view from the clinical perspective, confirming that acupuncture can significantly increase the levels of CD3+ and CD4+ T cells in patients with malignant tumors and improve prognosis (Wang et al. 2025). The immunomodulatory and synergistic mechanisms of acupuncture in reversing anti-PD-1 resistance are

speculative hypotheses summarized from existing evidence rather than directly validated by our current sequencing data. We propose several testable predictions for future validation: (1) Acupuncture may remodel the immunosuppressive tumor microenvironment and downregulate CD99-related inhibitory signaling in NSCLC patients; (2) Acupuncture intervention may improve the responsiveness of PD-1 inhibitors by reversing extrinsic adaptive immune resistance; (3) Combined acupuncture and anti-PD-1 treatment may optimize T-cell immune function and achieve a synergistic anti-tumor effect. Further *in vitro* experiments, animal models, and clinical trials are required to verify the mentioned mechanistic assumptions.

DISCUSSION

We utilized single-cell sequencing technology to investigate the remodeling effect of acupuncture combined with PD-1 inhibitor therapy on the tumor microenvironment of advanced NSCLC. Our study showed a significant reconfiguration of the immune microenvironment: the proportion of T cells and CD8+ cytotoxic T cells nearly doubled, while the proportion of tumor cells significantly decreased, indicating the generation of a potent and effective anti-tumor immune response (Riaz et al. 2017; Tumeh et al. 2014). This is consistent with current understanding of successful immune checkpoint inhibitor therapy (Huang et al. 2017; Yost et al. 2019). Additionally, our analysis also showed a deeper level of interaction complexity: we observed a high level of CD99-CD99 pairing between T cells and tumor cells.

Based on the recent clarification of the PILRα-CD99 axis, this discovery provided crucial clues for our interpretation (Xia et al. 2025). The study confirmed that CD99 on T cells acts as a receptor, binding to the PILRα ligand on tumor cells and transmitting inhibitory signals, thereby suppressing T cell function (Lu et al. 2014; Sun et al. 2012). This interaction may represent an 'adaptive resistance' mechanism, where tumors under immune attack upregulate inhibitory signals to counter the infiltration of effector T cells (Ribas 2015; Spranger et al. 2013). Therefore, the high expression of CD99-CD99 we observed is likely not indicative of efficient immune synapse formation, but rather a persistent attempt by residual tumor cells to impose immune checkpoint control and evade clearance (Gubin et al. 2014; Sade-Feldman et al. 2019).

We assume that in this combined treatment protocol, the key contribution of acupuncture lies in enhancing the function of T cells, enabling them to resist the inhibition mediated by PILRα-CD99. This assumption is supported by an increasing number of literatures on the immunomodulatory effects of acupuncture (Kavoussi & Evan Ross 2007; Zhang et al. 2014). Then, acupuncture has been proven to directly enhance the function of T cells. In a breast cancer model study, it was demonstrated that combining acupuncture with PD-1 inhibitors significantly enhanced CD4+ and CD8+ T cell infiltration and was associated with enrichment in T cell activation pathways, as shown by RNA sequencing analysis (Xu et al. 2025). Electro-acupuncture at the Zusanli acupoint could significantly increase the levels of interferon-γ and IL-2 in the serum, and enhance the expression of CD4+ T cells through the TRPV channel (Chen et al. 2017). This functional enhancement may enable T cells to have sufficient signal transduction resilience to overcome the inhibitory signals transmitted through CD99 (Sharpe & Pauken 2018; Thommen & Schumacher 2018).

Secondly, acupuncture can reshape the immunosuppressive tumor microenvironment. Acupuncture can restore the ability of immune homeostasis, including enhancing the function of CD8+ T cells in cancer and rebalancing the ratios of Th1/Th2 and Th17/Treg (Liu et al. 2024). Electro-acupuncture combined with PD-1 blockade can reprogram the immune microenvironment of colorectal cancer, presenting an enhanced cytotoxic feature (Wang et al. 2024). This 'immune normalization' effect is likely to extend to improving the metabolic adaptability of T cells, which is crucial for their maintaining effector functions in the nutrient-poor and inhibitory-signaled tumor microenvironment - this factor may be related to the enhanced Oxidative phosphorylation of tumor cells that we previously observed (Buck et al. 2017; O'Sullivan et al. 2019).

The synergy we proposed is thus a 'two-pronged' attack strategy. PD-1 blockade disrupts the PD-1/PD-L1 axis, releasing the 'brake' that causes T cell exhaustion (Okazaki & Honjo 2007; Pardoll 2012). At the same time, acupuncture provides a 'boost' to T cells, enhancing their intrinsic functions and metabolic adaptability, which in turn makes them less sensitive to the alternative 'brake' imposed by the PILRα-CD99 axis (Blank et al. 2019; Wherry & Kurachi 2015).

Our findings have significant clinical implications. They indicate that the combined use of acupuncture and immune checkpoint inhibitors may be a feasible strategy to enhance the treatment response rate, especially for patients who may have a risk of resistance (Minchom et al. 2016). As previous studies have shown, the potential synergistic effect with anti-PILRα antibodies also opens up new avenues for combining acupuncture with the next generation of immunotherapies.

Our study provides a molecular-level theoretical basis for the recently observed benefits in clinical observations (Marin-Acevedo, Kimbrough & Lou 2021). However, there are also some limitations. As a clinical study that integrates single-cell RNA sequencing analysis, its sample size is limited. Furthermore, the exact molecular mechanisms by which acupuncture enhances the resistance of T cells to CD99-mediated

inhibition remain to be clarified in clinical models. Future investigations should prioritize the identification of the key signaling interactions between acupuncture stimulation, T cell metabolism, and the PILR α -CD99 axis.

Our findings provide single-cell evidence that acupuncture may enhance the effectiveness of PD-1 inhibitors in strengthening antitumor immunity in advanced NSCLC. We propose a novel mechanism in which acupuncture improves the functional adaptability of T cells, thereby allowing them to overcome the immunosuppressive PILR α -CD99 checkpoint axis driven by the tumor. This study not only lays the groundwork for a promising integrative oncology approach but also underscores the potential of targeting T cell adaptability as a strategy to counteract emerging immune resistance. However, the synergistic regulatory effect of acupuncture on tumor immune microenvironment and immunotherapy resistance described in this study is an integrated hypothesis summarized from existing published evidence and bioinformatics analysis. It should be emphasized that such synergistic mechanism has not been directly verified by the current single-cell sequencing data. The present results only provide preliminary mechanistic implications and theoretical clues. Further biological experiments and clinical validation are required to confirm the exact synergistic effect and underlying regulatory mechanism.

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