

Unveiling chemotherapy-induced immune landscape remodeling and metabolic reprogramming in lung adenocarcinoma by scRNA-sequencing

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eLife Assessment

This study reports single-cell RNA sequencing results of lung adenocarcinoma, comparing 4 treatment-naïve and 5 post-neoadjuvant chemotherapy tumor samples. Of interest is the delineation of two macrophage subtypes: Anti-mac cells (CD45+CD11b+CD86+) and Pro-mac cells (CD45+CD11b+ARG+), with the proportion of Pro-mac/pro-tumorigenic cells significantly increasing in LUAD tissues after neoadjuvant chemotherapy. In terms of significance, the findings might be **useful**. However issues remain after the revision with lengthy descriptive clustering type analysis, insufficient statistical support, and inefficient figure presentation. As it stands, the level of supportive evidence is **inadequate**.

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Abstract

Background

Chemotherapy is widely used to treat lung adenocarcinoma (LUAD) patients comprehensively. Considering the limitations of chemotherapy due to drug resistance and other issues, it is crucial to explore the impact of chemotherapy and immunotherapy on these aspects.

Methods and Materials

Tumor samples from nine LUAD patients, of which four only received surgery and five received neoadjuvant chemotherapy, were subjected to scRNA-seq analysis. In vitro and in vivo assays, including flow cytometry, immunofluorescence, Seahorse assay, and tumor xenograft models, were carried out to validate our findings.

Results

A total of 83,622 cells were enrolled for subsequent analyses. The composition of cell types exhibited high heterogeneity across different groups. Functional enrichment analysis revealed that chemotherapy drove significant metabolic reprogramming in tumor cells and macrophages. We identified two subtypes of macrophages: Anti-mac cells (CD45+CD11b+CD86+) and Pro-mac cells (CD45+CD11b+ARG+) and sorted them by flow cytometry. The proportion of Pro-mac cells in lung adenocarcinoma tissues increased significantly after neoadjuvant chemotherapy. Pro-mac cells promote tumor growth and angiogenesis and also suppress tumor immunity. Moreover, through analyzing the remodeling of T and B cells induced by neoadjuvant therapy, we noted that chemotherapy ignited a relatively more robust immune cytotoxic response towards tumor cells.

Conclusion

Our study demonstrates that chemotherapy induces metabolic reprogramming within the TME of LUAD, particularly affecting the function and composition of immune cells such as macrophages and T cells. We believe our findings will offer insight into the mechanisms of drug resistance and provide novel therapeutic targets for LUAD in the future.

Introduction

Lung cancer is the most common cancer among all human tumor types, with more than 1.7×10^6 new cases worldwide each year. According to the Global Cancer Report data, lung adenocarcinoma (LUAD) accounts for most lung cancers (1 [↗](#)). The application of adjuvant or neoadjuvant chemotherapy (NCT) has significantly improved the long-term survival of LUAD patients. At present, for most LUADs that need chemotherapy after being assessed, chemotherapy will be used before and after surgery (2 [↗](#)). However, chemotherapy drugs are highly toxic and can often become ineffective (3 [↗](#)). In addition, continued ineffective chemotherapy will lead to the generation of drug-resistant tumor cell clones (4 [↗](#), 5 [↗](#)) and a delay in tumor removal. Almost all cancer patients show inherent or acquired drug resistance, leading to treatment failure and unsatisfactory overall survival. Therefore, to accurately develop therapies that can overcome drug resistance, it is essential to understand the alterations in the tumor microenvironment driven by chemotherapy.

Many studies have increasingly proved the tumor microenvironment (TME) to be an essential source of intratumoral heterogeneity (6 [↗](#)). The heterogeneity within the tumor microenvironment (TME) encompasses not only the variations between different tumor cells but also among various stromal and immune cell types. Investigating the dynamic changes in multiple cell populations within the TME of LUAD following chemotherapy may provide crucial insights

into overcoming chemotherapy resistance in LUAD. In this study, we demonstrated the changes in the microenvironment of lung adenocarcinoma with chemotherapy. In particular, we focused on the effect of chemotherapy on the metabolic reprogramming of tumor cells, stromal cells, and immune cells.

Formerly, it was generally believed that consuming glucose in TME by cancer cells may promote nutritional competition, a metabolic mechanism of immunosuppression (7 [↗](#)). However, recent studies have shown that tumor-infiltrating immune cells rely on glucose for their energy needs and functionality, with immune cells, particularly macrophages, consuming more glucose than malignant cells. The impaired immune cell metabolism in the tumor microenvironment (TME) helps tumor cells escape immunity (8 [↗](#)). The internal metabolic changes in the cells drive immune cells and cancer cells to preferentially obtain glucose and glutamine. It is believed that the selective cellular allocation of these nutrients can be used to develop therapeutic and imaging strategies to enhance or monitor the metabolic processes and activities of specific cell populations in TME (9 [↗](#)). Metabolic reprogramming in various cell types in the tumor microenvironment after undergoing chemotherapy may be an essential feature that affects chemotherapy. Our research fully demonstrated the metabolic reprogramming landscape of tumor cells, stromal cells, and immune cells before and after chemotherapy.

Materials and methods

Patients

All patients included in this study understood and signed written informed consent, (Approval number: B2019-436). The clinical samples of scRNA-seq came from patients diagnosed with LUAD, of which 4 cases received no treatment before surgery, and 5 cases received chemotherapy (Pemetrexed + Cisplatin). These samples were donated by inpatients in the Department of Thoracic Surgery, Zhongshan Hospital of Fudan University. After the lung adenocarcinoma tissue sample was taken, a small part was cut for paraffin sections, and the remaining tissue was dissociated into a single-cell suspension. 1×10^6 cells were drawn from the single-cell suspension for single-cell RNA sequencing.

Preparation of single-cell suspensions

For each patient, as described above, we dissociated the lung adenocarcinoma tumor sample into a single-cell suspension and then took 1×10^6 cells for single-cell RNA sequencing. We used the Tumor Dissociation Kit (Miltenyi Biotec, Gladbach, Germany) to digest tumor tissues with enzymes according to the manufacturer's instructions. In short, we first cut the lung adenocarcinoma tissue sample into small tissue pieces about 1 cm^3 with a surgical scalpel. We then transferred these small tissue pieces to the MACS C tube containing 4.7 mL DMEM serum-free medium, 200 μL Enzyme H, 100 μL Enzyme R, and 25 μL Enzyme A. After the tissue was incubated and digested in a constant temperature incubator 37 [for 1 hour, the tissue was mechanically separated by the MACS™ instrument. This procedure was repeated twice. After the tissue sample was dissociated, the sample was filtered with a 40 μm filter to remove the remaining large particles from the single-cell suspension. Centrifuge the suspension at $300 \times g$ for 7 minutes, then discard the supernatant.

Next, we used red blood cell lysate (10 $\times$) (Sigma-Aldrich, St. Louis, MO, USA) to remove red blood cells from the single-cell suspension. In short, add 1x Lysis Buffer to the centrifuge tube containing the single-cell pellet described above. The cell suspension was then incubated at room temperature for 15 minutes. To improve the quality of our samples, we also used a Dead Cell Removal Kit (Miltenyi Biotec) to ensure that the cell survival rate of our sequencing samples was >90%.

The 10x scRNA-seq data analysis

The R version used in our scRNA-seq data analysis study is 3.6.1. The cell quality control criteria are as follows: 1) The number of expressed genes is less than 300 or greater than 5000; 2) 10% or more of UMI is localized to mitochondrial or ribosomal genes. If they meet one of the criteria, the cells are excluded. After quality standardization, we applied the Seurat R package ([10](#)) to analyze the scRNA-seq data. First, we convert the scRNA-seq data into Seurat identifiable objects, and then we use the “FindVariableFeatures” function to find the first 2000 highly variable genes. After that, we applied principal component analysis (PCA) to reduce the dimensionality of scRNA-seq data. The “RunTSNE” function is used to perform t-distributed random neighborhood embedding (TSNE) to visualize various types of cells. The “FindClusters” and “FindAllMarkers” functions are used for cluster analysis of cell subclusters and detection of marker genes of cell subclusters.

Finally, according to the SingleR package ([11](#)), the CellMarker (<http://bio-bigdata.hrbmu.edu.cn/CellMarker/>) data set, and a previous report ([12](#)), we annotated different cell types. Simultaneously, some new potential marker genes were verified through experiments.

Analysis of Sub-Clusters of Cells in LUAD

After preliminary classification and annotation of all cells, epithelial cells, stromal cells, and immune cells are extracted through the “SubsetData” function. Then, we apply the “FindClusters” and “FindAllMarkers” functions to find the marker genes of each cell and perform dimensionality reduction clustering on each extracted cell through TSNE. The sub-clusters are annotated by dominantly expressed cell markers published by previous researchers. To select the marker genes that meet the requirements, we set the following cut-off thresholds to reveal the marker genes of each cluster: adjusted P-value <0.01 and multiple Log2FC >0.5.

Estimation of the copy number variations

To estimate the initial copy number variation (CNV) of each region, the R package “scCancer” ([13](#)) was applied. The expression level of each cell was used as the original input file for calculating CNV. Immune cells served as a background reference for calculating the CNVs scores of other cells. In addition, the R package “inferCNV” was used to quantify CNV in tumor cells as described previously ([14](#)).

Definition of cell scores and signature

To evaluate the M1/M2 polarization state and pro-/anti-inflammatory potential of macrophages, we performed a GSVA (Gene Set Variation Analysis) analysis. We retrieved gene sets related to the above functions from previous studies ([15](#)) and used them as references in this analysis.

We used the average expression of a published list of characteristic genes for T cell toxicity and exhaustion to define T cells’ cytotoxicity, exhaustion, and costimulation scores.

Identification of gene markers of malignant cells

We used the identified malignant cell marker genes in tumor cells to identify gene expression characteristics in malignant cells. Then, we performed unsupervised NMF (Non-negative Matrix Factorization) to reveal the malignant characteristics of tumor cells through the NMF R package ([16](#)).

Trajectory analysis

We used the monocle2 R package to analyze the trajectory of all cells to explore the trajectory progression of various types of cells in a single cell (17 [↗](#)). First, apply the function “newCellDataSet” to construct a data object that the monocle 2 R package can recognize. Afterward, the differentially expressed genes identified by the Seurat R package were selected for cell trajectory analysis. The “reduceDimension” function was used to reduce the dimensionality. We used the “orderCells” function to project cells on a pseudo-time trajectory to show the trend of cell evolution. A state consisting of cells mainly derived from nonmalignant tissues in a cluster identified as epithelial cells was defined as “root cells.”

Analyses of metabolic pathways

To evaluate the activity of various metabolic pathways of each cell type, we applied the algorithm developed by Xiao et al. (18 [↗](#)). In short, the analysis of metabolic programs is based on the average expression level of metabolic genes across cell types to indirectly reflect the metabolic activity of cells.

A variety of environmental factors may potentially affect the metabolic reprogramming of tumors, such as chemotherapy, nutrient supply, and the environment where the cells are located. Therefore, exploring these factors and the cross-conversion between glycolysis and mitochondrial activity in various cells in the tumor microenvironment is essential for understanding the metabolic reprogramming of tumors.

We calculated the average gene expression levels in glycolysis and OXPHOS as indicators of glucose supply and mitochondrial activity, respectively. The data of genes that were responsive to the two groups of genes (known to be responsive to glycolysis and OXPHOS) used in the calculations were retrieved from the MsigDB database. At the same time, the cells were sorted by flow cytometry, and the contents of various metabolites were tested, in turn, to verify whether they were consistent with gene expression levels.

Cell Interaction Network analysis

To study the cell-to-cell interactions between tumors and nonmalignant cells, immune cells, and stromal cells, we applied the R package “CellChat” (19 [↗](#)) and “CellPhoneDB” Python package for analysis (20 [↗](#)). The crosstalk analysis between cells through the “CellChat” package was as follows: (1 [↗](#)) First, use the “createCellChat” function to create a data set object that can be identified by “CellChat”; (2 [↗](#)) Then use “aggregateNet”, “computeCommunProbPathway”, and “computeCommunProb” function to automatically infer the possible cellular communication network between cells; (3 [↗](#)) Finally, the “netVisual_aggregate”, “netVisual_bubble” and “netVisual_signalingRole” functions were used to visualize the interaction between these cells. Then use the built-in parameters to apply the “CellPhoneDB” R package.

Immunohistochemistry and immunofluorescence

The paraffin-embedded lung cancer tissue sections were deparaffinized with xylene and rehydrated. Discard the blocking solution, add the primary antibody, and incubate overnight at 4 degrees. After removing the primary antibody and washing thoroughly, add the secondary antibody to incubate for 1 hour, and then add DAB chromogenic reagent (Gene Tech, China) for color development. Finally, hematoxylin is used for nuclear dyeing.

As mentioned in the above immunohistochemistry experiment, the steps before incubating the primary antibody are the same. Incubate with the corresponding primary and secondary antibodies with green and red fluorescent dyes, respectively, and then use DAPI to stain the nuclei.

Flow cytometry assay

Cells and APC-conjugated mouse anti-human CD45, FITC-conjugated mouse anti-human CD11b, BV421-conjugated mouse anti-human ARG1, as well as pe-cy-conjugated mouse anti-human CD86 (5 $\mu\text{L}/10^6$ cells; BD Biosciences) were incubated on ice for 30 minutes. Then, FACSria III (BD Biosciences) was used to quantify the required cells, and FlowJo software (TreeStar, Woodburn, OR, USA) was used to analyze the results.

Animal experiments

All animals involved in this study were treated humanely and received standard care. The animal experimental procedures were approved by the Institutional Review Board of Zhongshan Hospital of Fudan University (Shanghai, China). In this experiment, we housed male athymic nude mice (BALB/cASlac-nu) in a specific pathogen-free environment. We mixed treated A549 cells and TAMs to make a 1:1 cell mixture at a cell concentration of 5×10^6 cells/ml. Take 0.05ml of the mixed suspension of cells and Matrigel, and implant them into the lung thoracic cavity of nude mice for in situ tumor formation experiments.

Animals were sacrificed when one of the following signs of disease was observed: tumor ulceration (greater than 0.5 cm); inability to move or eat; or serious injury. Changes in tumor size were detected using an optical imaging system for in vivo small animals (IVIS Spectrum, PerkinElmer, USA).

Statistical Analysis

The statistical tools, methods, and thresholds of each analysis are clearly described in the results or detailed in the legend or materials and methods.

Results

Single-cell transcriptomic profiling of LUAD

A total of 9 patients with non-metastatic LUAD underwent lobectomy with curative intent in the Department of Thoracic Surgery, Zhongshan Hospital of Fudan University. Among them, five received three cycles of preoperative neoadjuvant combination chemotherapy with cisplatin plus pemetrexed (defined as NCT group), while others only received surgery (defined as the Control group). Following resection, a malignant lung tumor sample was obtained from each patient, rapidly digested to a single-cell suspension, and analyzed using 10X scRNA-seq (**Figure 1a** [↗](#)). After quality control, a total of 83,622 cells that met the inclusion criteria were subjected to subsequent analyses, with 33,567 and 50,055 cells derived from the control and NCT groups, respectively (**Figure 1a-c** [↗](#), S1a). Next, we classified cell types through dimensional reduction and unsupervised clustering using the Seurat package and relative marker genes.

Using the SingleR package, the CellMarker dataset, and our previous studies ([21](#) [↗](#), [22](#) [↗](#)), we identified cell clusters that could be assigned to known cell lineages: epithelial cells (marked by SFTA2 and KRT8), T cells (marked by CD3D and TRBC2), B cells (marked by CD79A and CD19), endothelial cells (marked by EMCN and CXorf36), mast cells (marked by TPSB2 and TPSAB1), macrophages (marked by CD68 and APOE), monocytes (marked by FGL2 and LGALS2), fibroblasts (marked by LUM and DCN), neutrophils (marked by FCGR3B and CMTM2). Meanwhile, the consensus clustering of these cells also exhibited the consistency and homogeneity of the expression profile within each identified cell type (**Figure 1b** [↗](#)). For instance, clusters 1, 3, 6, 7,

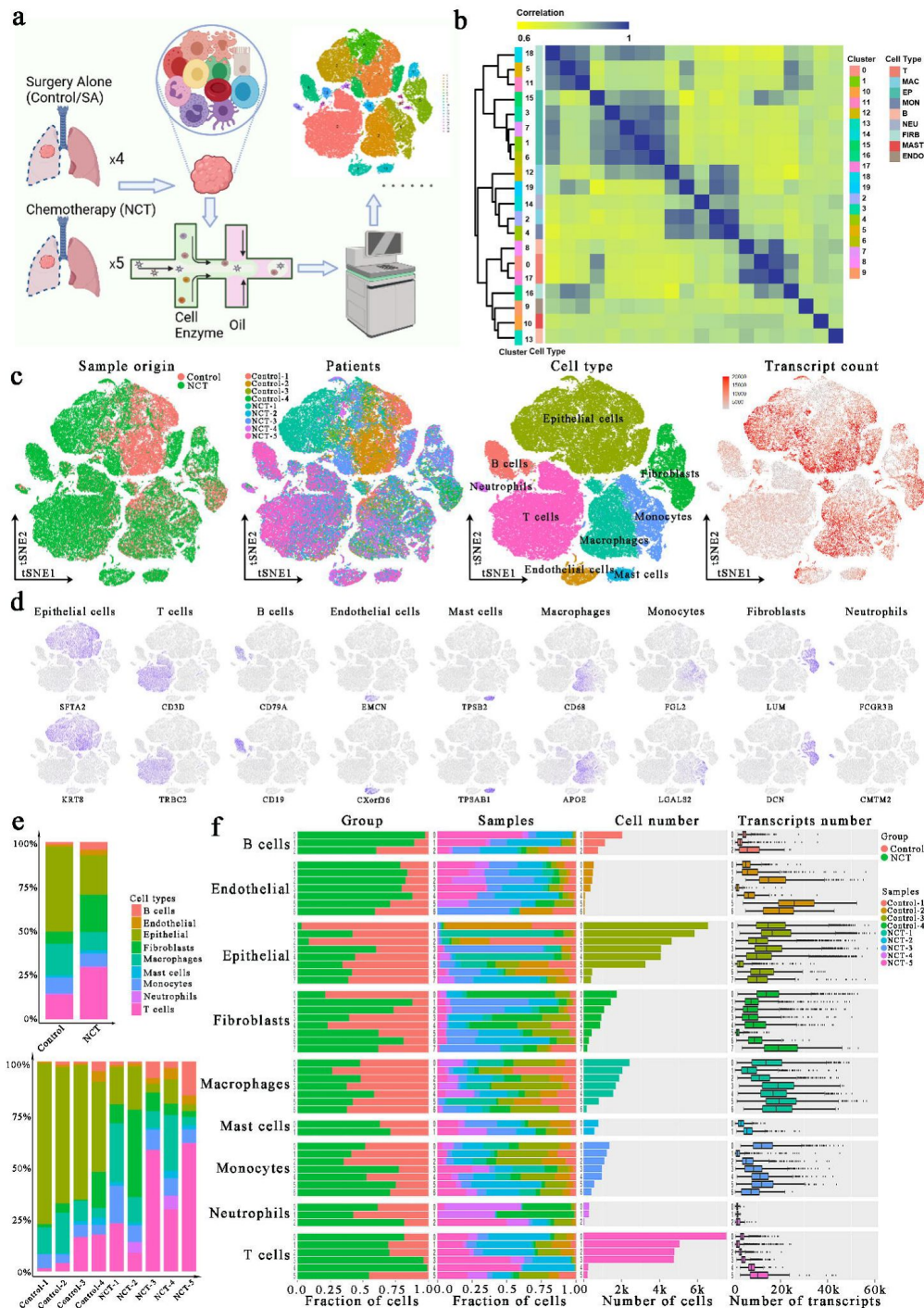


Figure 1.

Single-cell atlas of lung adenocarcinoma (LUAD) tissues from the control, and NCT group. **(a)** Workflow depicting collection and processing of LUAD samples for scRNA-seq analysis. **(b)** Consensus clustering based on the correlations among the 20 clusters identified through the tSNE algorithm. **(c)** tSNE of the 83622 cells enrolled here, with each cell color indicating: its sample type of origin, the corresponding patient, predicted cell type, and the transcript counts. **(d)** Expression of marker genes for the cell types defined above each panel. **(e)** The proportion of each cell type in different groups and samples. **(f)** For each of the eight epithelial subclusters and 43 non-epithelial clusters (left to right): the fraction of cells originating from the three groups, the fraction of cells originating from each of the nine patients, the number of cells and box plots of the number of transcripts (with plot center, box, and whiskers corresponding to the median, IQR and $1.5 \times \text{IQR}$, respectively). NCT: Neoadjuvant chemotherapy.

and 15, all designated as epithelial cells, were adjacent to each other in the consensus heatmap. This result confirms the robustness and reliability of our data pre-processing. Detailed distributions of these marker genes in each cluster are depicted in Figure S1.

By comparing the composition of different types of cells in each group, we noticed tumor microenvironment heterogeneity: the proportion of cells other than tumor cells, especially immune cells (mainly T and B), was significantly higher in the NCT (**Figure 1e**). Therefore, to identify subclusters within each of these nine major cell types, we observed a complex cellular ecosystem containing eight different epithelial subclusters and 43 non-epithelial clusters. Interestingly, the epithelial subclusters, mainly composed of cancer cells, were highly patient-specific, while the immune cell subclusters mostly consisted of cells derived from four or more patients (**Figure 1f**). This observation demonstrated the substantial variation and heterogeneity of tumor microenvironment among groups and individuals. Therefore, we further explored these alterations associated with the therapeutic regimen in greater detail for the primary cell types in subsequent analyses.

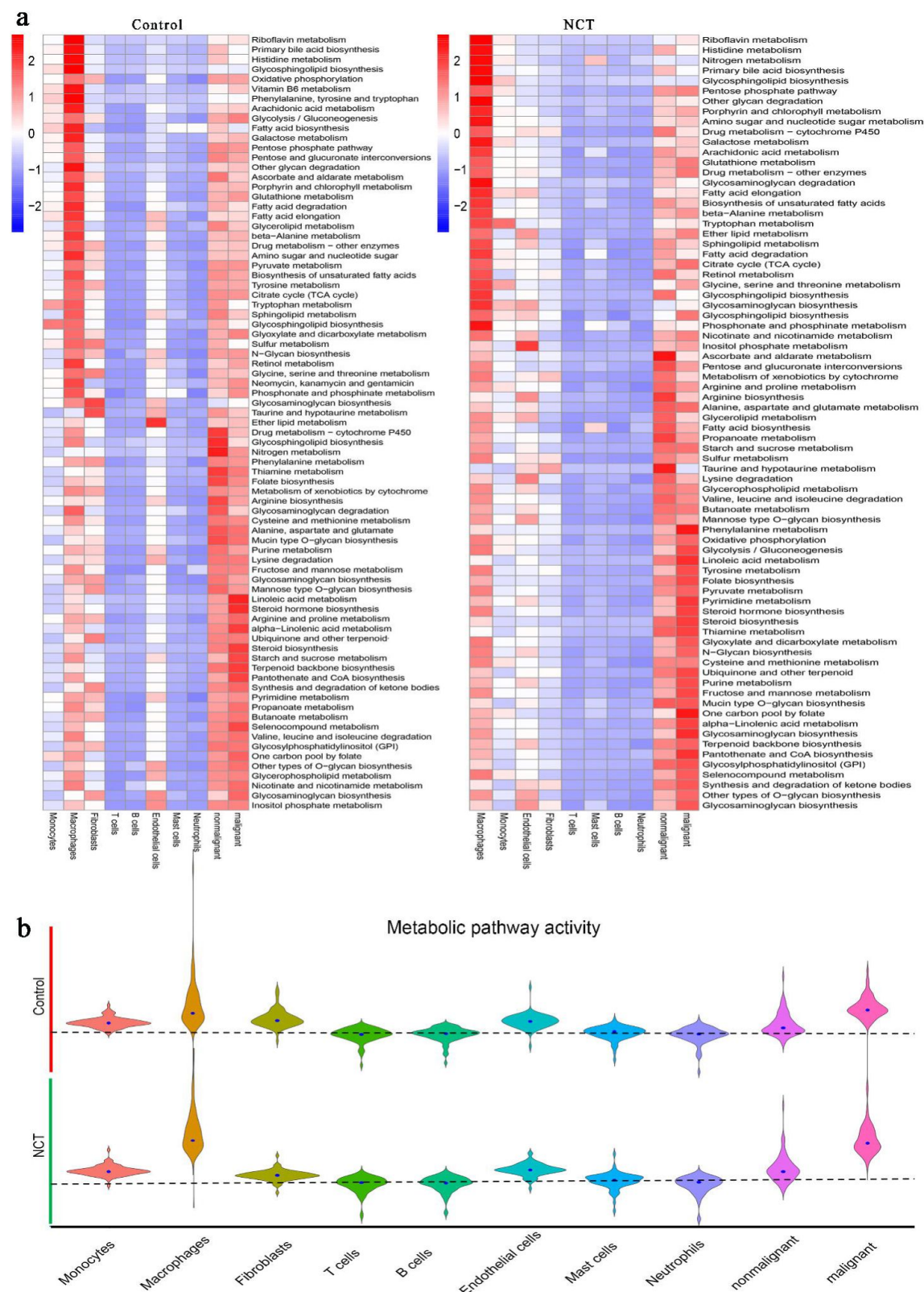
Metabolic reprogramming in lung adenocarcinoma driven by neoadjuvant chemotherapy

Metabolic reprogramming is a hallmark of malignant tumors. Recent studies have also shown that tumors' metabolic characteristics and preferences change during cancer progression (23). In each type of cell derived from the Control and NCT groups, more significantly up-regulated metabolic pathways were enriched in cancer cells, nonmalignant epithelial cells, fibroblasts, and macrophages (**Figure 2a**). The enrichment of oxidative phosphorylation, glycolysis, pyruvate metabolism, and the tricarboxylic acid cycle indicates active glucose metabolism in these four cell types. By analyzing the activity of metabolic pathways in cells from different sources, we found that the activity scores of the metabolic pathways of tumor cells and macrophages were significantly higher than those of other types of cells. Notably, the metabolic pathway activity of macrophages and malignant cells increased after chemotherapy (**Figure 2b**).

Changes in metabolism and gene expression of tumor cells after neoadjuvant chemotherapy

To accurately analyze the effect of chemotherapy on the cancer cells, we first re-clustered the epithelial cells, and 12 clusters were identified (**Figure 3a**). Copy number variations (CNVs) (Figure S2a) and marker genes were used to accurately separate malignant and nonmalignant epithelial cells in Control and NCT samples. They were finally defined as MalignantSA cells (Marker genes: FOXL2/MET/CD74), MalignantNCT cells (Marker genes: RAC1/MAF/CXCL1), and Nonmalignant cells (Marker genes: ABCA3/SFTPB/LPCAT) (**Figure 3d**). These marker genes were further confirmed by immunofluorescence experiments (Figure S2d). We found that the proportion of malignant cells was significantly reduced after chemotherapy (**Figure 3b, c**, Figure S2b). Although malignant cells were significantly reduced after chemotherapy, genetic aberrations by CNVs analysis revealed that MalignantNCT cells exhibited significantly higher malignant scores compared to MalignantSA cells (Figure S2a).

We performed trajectory analysis to track the reprogramming of epithelial cells across the three groups. Nonmalignant cells evolved in two directions and developed into two clusters of cells (**Figure 3e**). In this evolutionary process, glycolysis-related genes (ENO1, LDHB, GAPDH), oxidative phosphorylation-related genes (NDUFA4), mitochondrial repair-related genes (TOMM7), glucose and lipid metabolism regulation genes (S100A16), ATPase activity-related genes (CCT6A), tumor immune regulation-related genes (CCL20, CXCL1, PAEP, PPP1R14B), hypoxia response regulation genes (CHCHD2), apoptosis regulation genes (MEG3, CEACAM5), mRNA alternative splicing-related genes (LSM5), and Ras-related protein (RAC1, RALA) gradually increased over time



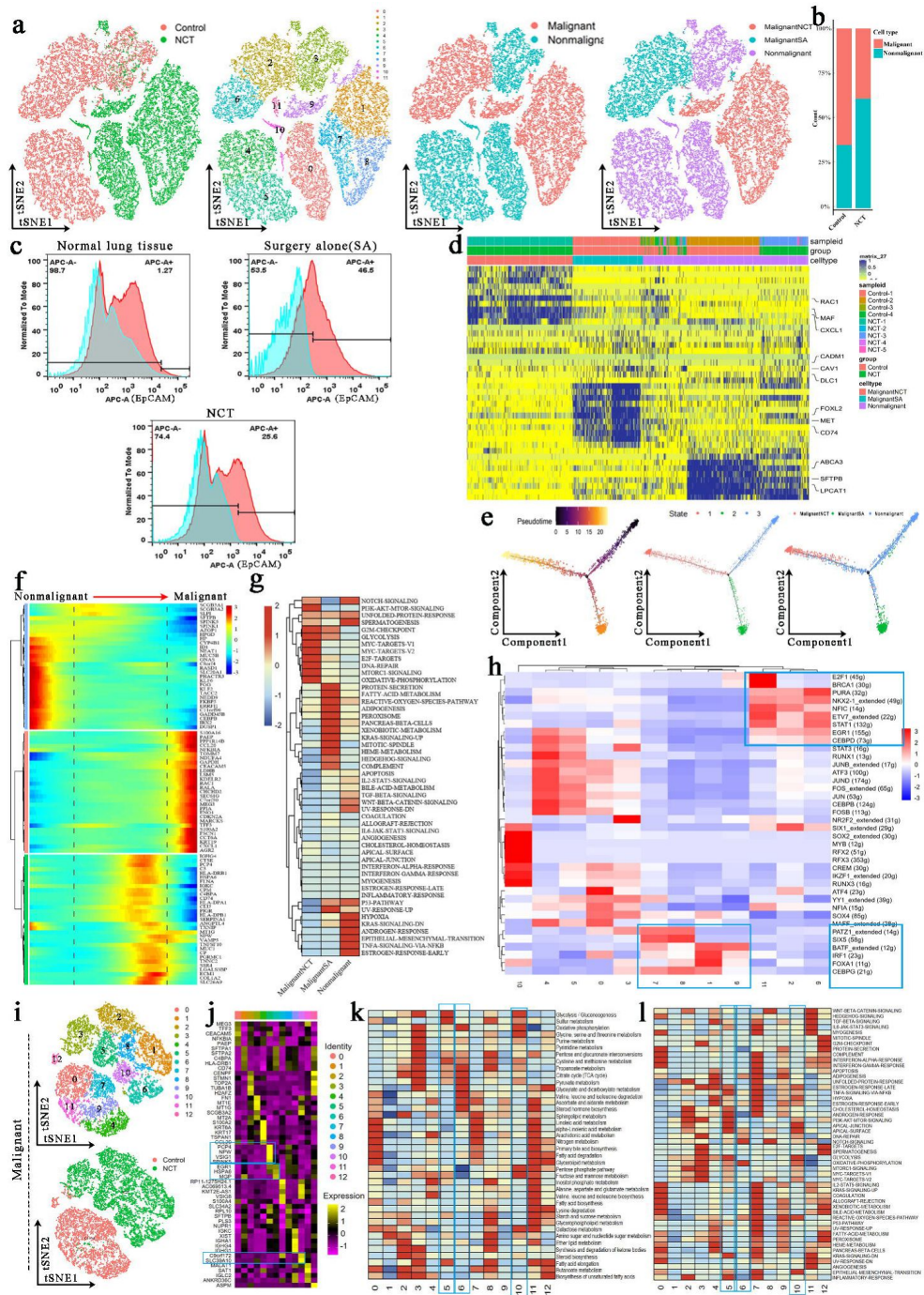


Figure 3.

Tumor cells and epithelial cells had significant phenotypic changes before and after chemotherapy. **(a)** The tSNE plots and overview of the tumor cells and epithelial cells. **(b)** The proportion of malignant cells and nonmalignant cells in the Control group and NCT group. **(c)** Flow cytometry showed that the proportion of malignant cells was significantly reduced after chemotherapy and immunotherapy. **(d)** Marker genes of MalignantSA cells, MalignantNCT cells, and Nonmalignant cells. **(e)** Pseudotime analysis showed that nonmalignant cells evolved in two directions. **(f)** The heat map showed that a series of genes play an important role in transforming epithelial cells into tumor cells. **(g)** GSEA analysis was performed for malignant and nonmalignant cells. **(h)** SCENIC analysis revealed the hub genes in the malignant transformation of epithelial cells. **(i)** The tSNE plots for re-clustered malignant cells. **(j)** Marker genes of 13 sub-clusters from malignant cells. **(k)** Metabolic characteristics in different malignant cell sub-clusters. **(l)** GSVA analysis reveals the characteristics of pathway activity in different malignant cell sub-clusters.

in the pseudotime analysis. These findings indicate that these genes play an essential role in the transformation of epithelial cells into tumor cells (**Figure 3f**, Figure S2e). Correspondingly, during the process of epithelial cells transforming into malignant tumor cells, the activity of the glycolysis pathway, oxidative phosphorylation pathway, angiogenesis pathway, DNA repair pathway, mTORC1 signaling pathway gradually increased over time. However, P53 pathway, apoptosis signaling pathway activity then steadily decreased (Figure S2f).

Similarly, we performed GSVA analysis on malignant and nonmalignant cells from the three groups. We found that the glycolysis pathway, oxidative phosphorylation pathway, MYC-targets, E2F-targets, DNA repair pathway, and mTORC1 signaling pathway were significantly enriched in MalignantNCT cells derived from the NCT group (**Figure 3g**). The metabolic reprogramming enables cancer cells to resist anti-cancer drugs, thereby developing chemoresistance (24). To find the hub genes that cause the malignant transformation of epithelial cells, through Single-Cell Regulatory Network Inference and Clustering (SCENIC) analysis, we found that E2F1, BRCA1, PURA, NKX2-1, NFIC, ETV7, STAT1, EGR1, and CEBPD were highly expressed in malignant cells (cluster 2, 6, 11) from the Control group. In contrast, the malignant cells from the NCT group (clusters 1, 7, 8, 9) have high expression of transcription factors (TFs) such as PATZ1, SIX5, BATF, IRF1, FOXA1, and CEBPG. After neoadjuvant chemotherapy, the increased expression of these TFs promoted the occurrence of lung adenocarcinoma complex phenotypic remodeling (**Figure 3h**).

Tumor cells have significant heterogeneity. We re-clustered the malignant cells and obtained 13 sub-clusters (**Figure 3i**). Cluster 1, 2, 5, 6, 8, and 10 were derived from the NCT group (**Figure 3i**). Through the analysis of the metabolism of these cell subclusters, we found that clusters 5 (marker genes: PCP4/NPW/VSIG1), 6 (marker genes: ERG1/HSPA6), and 10 (marker genes: C9orf172/SLC39A10) from the NCT group showed high levels of glycolysis, oxidative phosphorylation and pyruvate metabolism (**Figure 3k**). GSVA analysis also showed that the glycolysis and oxidative phosphorylation signaling pathway-related genes were significantly enriched in clusters 5, 6, and 10 (**Figure 3l**). Similarly, we re-clustered nonmalignant cells to obtain 16 sub-clusters, of which clusters 1, 3, 8, 11, 12, 13, 15 were from the NCT group, and the rest were from the Control group (Figure S2g, h). Clusters 4 and 7 from the Control group showed high levels of glycolysis and oxidative phosphorylation (Figure S2i, j), which contrasts with the glucose metabolism observed in malignant cells from the Control group.

Changes in stroma cells resulted from neoadjuvant chemotherapy

To investigate stromal cell dynamics in the tumor microenvironment (TME), we obtained 8944 presumed stromal cells, as shown in **Figure 1c**. We re-clustered them into five sub-populations, including COL14A1-positive fibroblasts, endothelial-1, endothelial-2, myofibroblasts, pericytes, and smooth muscle cells (SMC) (**Figure 4a-c**) (25–27). Detailed expression of the marker genes in each cell type is outlined in **Figure 4c**. Herein, we noticed a significant difference between the distribution of each of these five clusters in patients receiving varied types of treatment. The COL14A1-positive fibroblasts comprised the main fibroblast types in NCT groups, in which both endothelial 1 & 2 were mainly found. Pericyte and SMC were presented in all three groups. In contrast, myofibroblasts exclusively originated from the control group. According to previous research, myofibroblasts have been described as cancer-associated fibroblasts that participate in extensive tissue remodeling, angiogenesis, and tumor progression (25, 26). Therefore, this finding revealed that NCT and immunotherapy significantly altered the stromal cell composition in the tumor microenvironment.

To explore the activity of known biological pathways in these stromal cells, we performed functional enrichment analysis. In particular, GSVA analysis exhibited that endothelial 1 & 2 shared several up-regulated pathways related to cell proliferation and fate regulation, including IL6-JAK-STAT3, TGF β , and WNT- β catenin signaling. Besides, pathways associated with energy metabolisms such as glycolysis and hypoxia were up-regulated in myofibroblast, whereas pericyte was characterized by enriched oxidative phosphorylation and adipogenesis (**Figure 4d**).

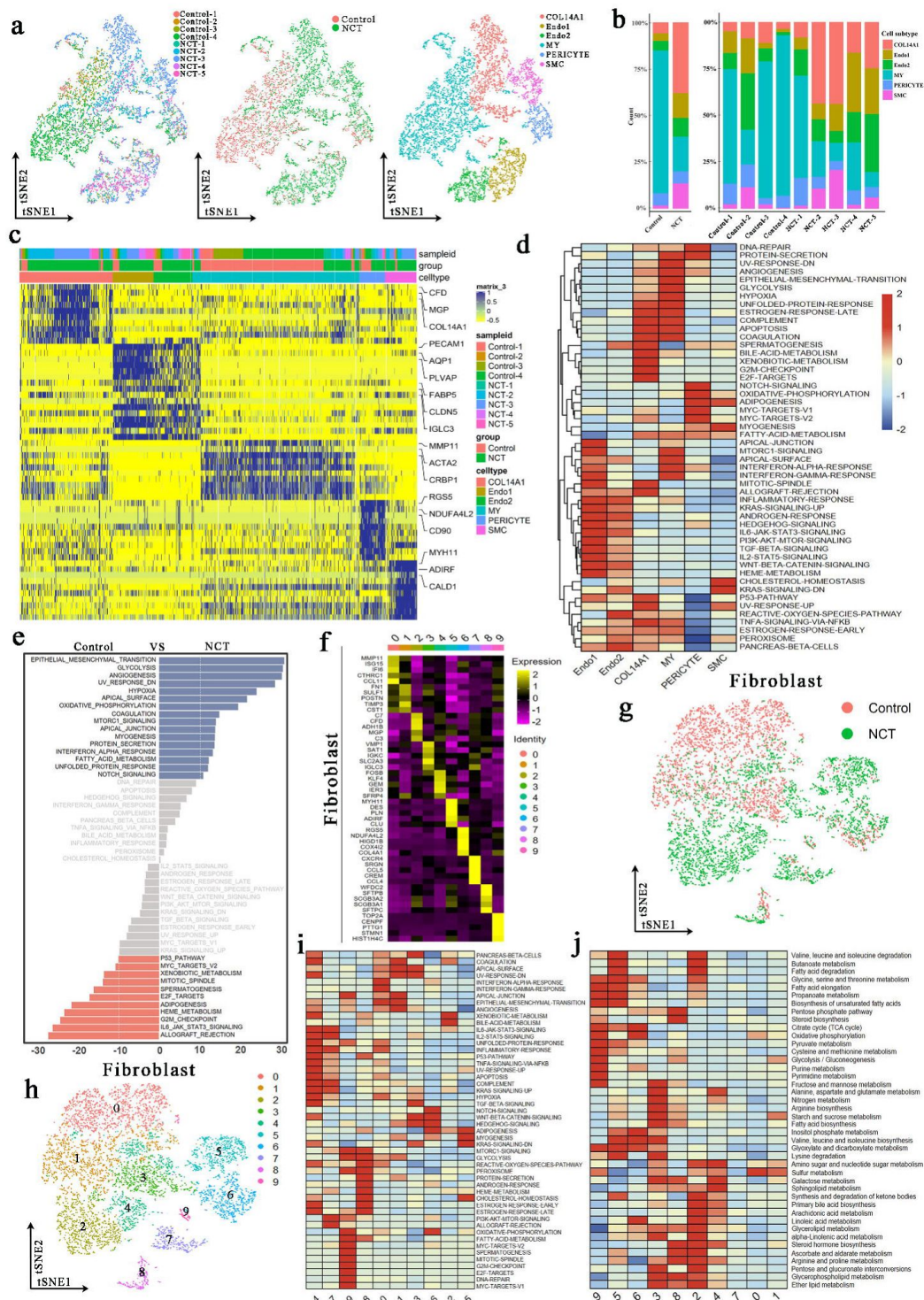


Figure 4.

The scRNA profile of stromal cells derived from LUAD samples in control and NCT groups. **(a)** The tSNE plots an overview of the 6 clusters of stromal cells. **(b)** Proportions of the six predicted clusters of stromal cells in different groups and samples. **(c)** Heatmap exhibiting the expression level of marker genes in each stromal cell cluster. **(d)** GSVA analysis estimated the pathway activation levels of different stromal cell subtypes. The scores have been normalized. **(e)** GSVA analysis revealed the activation level of hallmark pathways in stromal cells (control vs. NCT groups) **(f)** Heatmap exhibiting the expression level of marker genes in each fibroblast cluster. **(g-h)** The tSNE plots revealed the group origins **(g)** and predicted subclusters **(h)** of fibroblast. **(i-j)** GSVA analysis estimated the pathway activation levels of different fibroblast subtypes.

Meanwhile, when comparing the GSVA scores of these biological processes between patients from control or NCT groups, we noted that the stromal cells exhibited enhanced metabolic levels after NCT, as represented by up-regulated glycolysis, oxidative phosphorylation, and fatty acid metabolism pathways (**Figure 4e** [↗](#)).

Considering the essential role of fibroblasts and their complicated function in shaping the tumor microenvironment, we further re-clustered them into ten subgroups (**Figure 4f-h** [↗](#)). As shown in **Figure 4i-j** [↗](#), the GSVA score of the metabolic pathways, including glycolysis and oxidative phosphorylation, and pyruvate metabolism and citrate cycle (TCA cycle), were up-regulated in clusters 5, 6, and 9. Intriguingly, the upregulation of these pathways was mainly observed in NCT groups (Figure S3a). The three clusters were represented by distinct gene expression profiles, such as overexpressed MYH11 in cluster 5, RGS5 in cluster 6, and TOP2A in cluster 9. Since the potential involvement of these genes in the manipulation of fibroblast metabolism has never been proposed yet, they might serve as new specific markers of the fibroblast subtype with such a high metabolic rate in the tumor microenvironment. Besides, the SCENIC analysis demonstrated that MEF2C, NFIA, and RAD21 might drive the formation of these clusters, respectively (Figure S3c). Further in vitro studies are required to elucidate these notable fibroblasts' potential function and driver genes in LUAD's development and response to NCT. Conclusively, cellular dynamics in stromal cells support a consistent phenotypic shift of fibroblasts towards an increased metabolic level after preoperative chemotherapy.

Chemotherapy drove tumor-associated macrophages to turn more into phenotypes that promote tumor progression

In the process of cancer formation, tumor-associated macrophages (Tumor-Associated Macrophages, TAM) have an essential influence on the inflammatory response in the tumor microenvironment (28 [↗](#)). To study the effects of chemotherapy on TAMs, we first extracted all macrophages (10526 cells) and re-clustered them into ten cell clusters (**Figure 5a** [↗](#)). From **Figure 1e** [↗](#), we can see that the proportion of macrophages after chemotherapy was reduced.

The cell clusters derived from the Control group were 1/2/3/5/7 clusters, those from the NCT group were mainly 0/4/8 clusters, and the number of cells in the 6/9 clusters from the Control group and the NCT group was similar (**Figure 5a** [↗](#)). The proportion of cells in cluster 0 (marker genes: CXCL8/ CCL20/CHIT1), 4 (marker genes: CCL3/ CCL4/ SEPP1), 8 (marker genes: ARG2/ S100A2) decreased after chemotherapy, while the remaining cell clusters increased (**Figure 5b** [↗](#), c). Through the GSVA analysis, we found that glycolysis, angiogenesis, PI3K-AKT-mTOR-signaling, IL6-JAK-STAT3-signaling, hypoxia, TGF-beta-signaling, and other signaling pathways were significantly enriched in cluster 0/1/8. Promoting inflammation-related signaling pathways such as TNF-signaling-via-NFKB, inflammatory-response, Notch-signaling, fatty-acid-metabolism, and oxidative-phosphorylation were increased dramatically in clusters 2/4/7/9 (**Figure 5d** [↗](#)).

Similarly, we found that glycolysis/gluconeogenesis, amino sugar and nucleotide sugar metabolism, alanine, aspartate, and glutamate metabolism were more active in the 0/1/8 cluster. In contrast, oxidative phosphorylation, citrate cycle, pyruvate metabolism, fatty acid elongation, fatty acid biosynthesis, etc., were more active in clusters 2/4/7/9 (**Figure 5e** [↗](#)). According to the GSVA analysis, the glycolysis/gluconeogenesis signaling pathway was significantly enriched in macrophages from the NCT group. In contrast, macrophages from the Control group showed a high activity in oxidative phosphorylation, fatty acid elongation, fatty acid degradation, fatty acid biosynthesis, and citrate cycle (TCA cycle) (**Figure 5f** [↗](#)). These results indicate that significant metabolic reprogramming occurred in tumor-associated macrophages after chemotherapy, and different TAMs cell clusters also showed huge metabolic differences. In general, our results revealed that chemotherapy could promote glycolysis of TAMs and inhibit fatty acid metabolism.

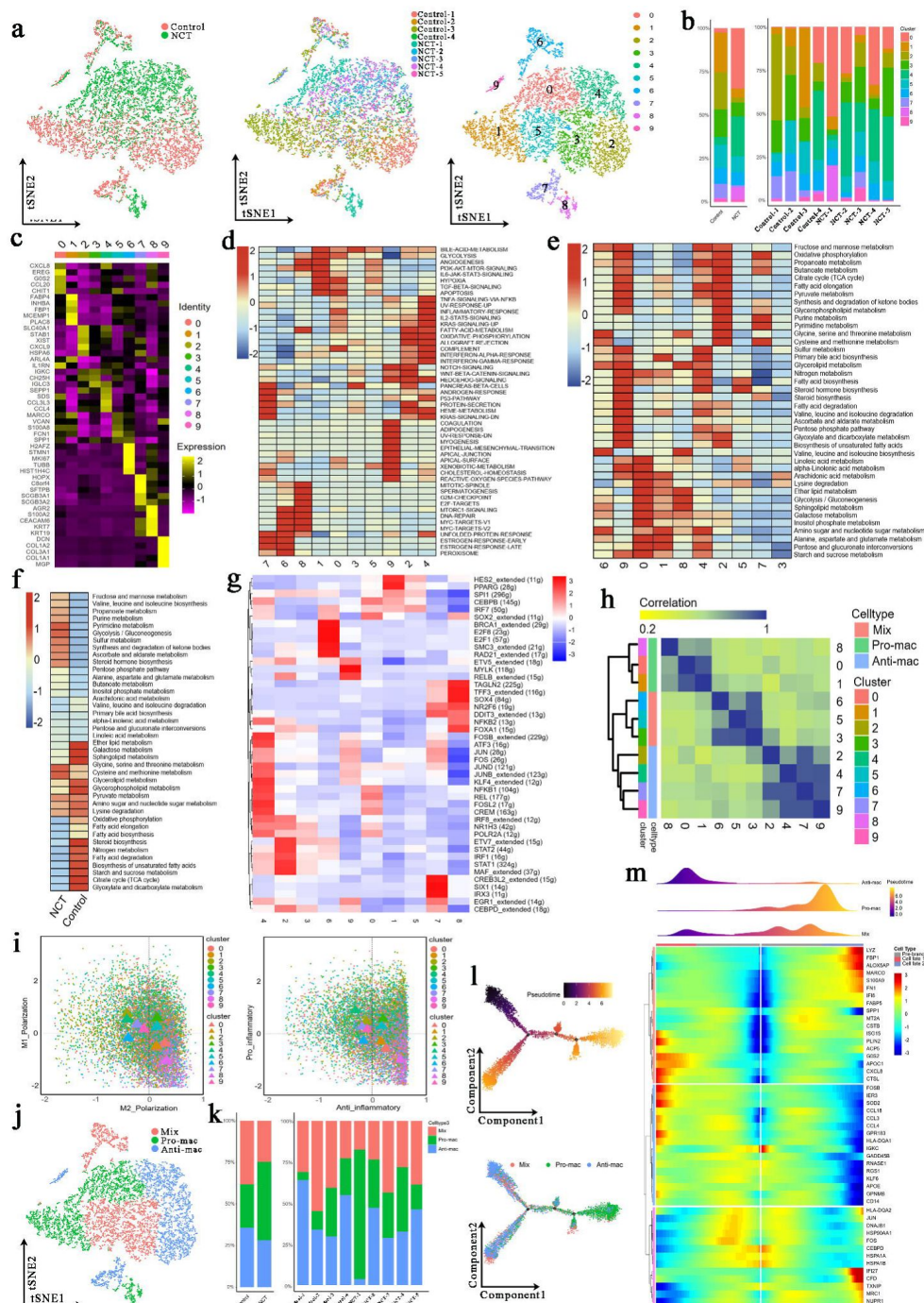


Figure 5.

Three newly identified subtypes of tumor-associated macrophages (TAMs) displayed distinct genetic and metabolic features. **(a)** The tSNE plots showed the group origins, sample origins, and clusters of TAMs. **(b)** Proportions of the 10 clusters of TAMs in different groups and samples. **(c)** Marker genes of the 10 clusters of TAMs. **(d)** GSVA analysis was performed for the 10 clusters of TAMs. **(e)** The activity of various metabolic processes in the 10 clusters of TAMs. **(f)** GSVA analysis was performed for TAMs from the Control group and NCT group. **(g)** SCENIC analysis was performed for the 10 clusters of TAMs. **(h)** Consensus clustering based on the correlations among the 10 clusters of TAMs identified through the tSNE algorithm. **(i)** Polarization score (left) and inflammatory score (right) for 10 clusters of TAMs based on the expression of polarization marker genes and inflammatory genes. **(j)** The tSNE plots for three types of TAMs. **(k)** Proportions of the three subtypes of TAMs in different groups and samples. **(l)** Development trajectory analysis for the three subtypes of TAMs. **(m)** Pseudotime analysis revealed a series of genes that affect the differentiation and development of macrophages.

To explore the key genes that regulate the differences in the metabolism of each subcluster of macrophages, we performed a SCENIC analysis. We found that HES, PPARG, SPI1, CEBPB, and IRF7 were highly expressed in cluster 0/1, which may be the key genes that regulate the conversion of macrophages into M2-like TAMs, while clusters 2/4/7/9 highly expressed STAT1, STAT2, NFKB1, JUN, and FOS that regulate the conversion of macrophages to M1-like TAMs (**Figure 5g** [↗](#)).

According to the gene expression of macrophages, we divided these 10 clusters of cells into three subtypes of macrophages through cluster analysis (**Figure 5h** [↗](#)). We scored the expression levels of pro-inflammatory and anti-inflammatory genes in all macrophages. We displayed each color-coded macrophage subtype's M1 and M2 scores (left) and pro-inflammatory and anti-inflammatory scores (right) through a scatter plot.

Similarly, we found that 0/1/8 cluster cells exhibited M2-like polarization and anti-inflammatory properties, while 2/4/7/9 exhibited M1-like polarization and pro-inflammatory properties (**Figure 5i** [↗](#)). Based on these analyses, we divided these 10 clusters of macrophage subtypes into three categories: M1-like polarized phenotype was defined as Anti-mac; M2-like polarized phenotype was defined as Pro-mac; those without obvious polarized phenotype were defined as Mix (**Figure 5j** [↗](#)). We found that the proportion of Pro-mac in the tumor microenvironment increased after chemotherapy, especially in the case of NCT-1 (**Figure 5k** [↗](#)). Interestingly, via trajectory analysis we found that two subtypes, Anti-mac and Mix, can be converted to Pro-mac. In this evolution process, the high expression of LYZ, FBP1, ALOX5AP, MARCO, S100A9, FN1, CXCL8, APOC1CTSL, and other genes may have played an essential role in promoting the conversion of Anti-mac to Pro-mac (**Figure 5l,m** [↗](#)). This suggests that we can change the phenotype of TAMs in the tumor microenvironment by altering the expression of these genes.

Chemo-driven Pro-mac and Anti-mac metabolic reprogramming exerted diametrically opposite effects on tumor cells

To further verify the remodeling effect of chemotherapy on the functional phenotype of TAMs in the tumor microenvironment, we first used the FindAllMarkers function in the Seurat package to find the marker genes of Pro-mac, Anti-mac, and Mix cells. Pro-mac was mainly characterized by high expression of CXCL8, ARG1, CREM, CD206, STAT6, CCL22, MMP7, and CCL3L3, while Anti-mac was mainly characterized by high expression of CD86, HLA-DR, PLAC8, CXCL10, COX2, IL15R, and SCGB3A1 (**Figure 6a** [↗](#)). Based on these marker genes, we sorted out Anti-mac cells (CD45+CD11b+CD86+) and Pro-mac cells (CD45+CD11b+ARG+) by flow cytometry (**Figure 6b** [↗](#)). To verify whether the cells we sorted were the cell population we wanted, we re-verified the positive rates of Pro-mac and Anti-mac cells by flow cytometry (**Figure 6c** [↗](#)). Our results showed that the proportion of Pro-mac cells in lung adenocarcinoma tissues after neoadjuvant chemotherapy increased significantly (**Figure 6d** [↗](#)). In fact, by performing immunofluorescence staining on lung adenocarcinoma tissue samples derived from surgery alone and neoadjuvant chemotherapy, we also found that the proportion of cells marked by the marker gene CD206 of M2-like TAMs increased significantly after chemotherapy (**Figure 6e** [↗](#)). Macrophages can promote tumor progression by secreting many cytokines. By analyzing the differentially expressed genes of Pro-mac and Anti-mac cells, we found IL10, PDCD1LG2, PDGF, VEGF, MMP9, CXCL9, CXCR4, IL22, KLF4, and TGF- β were highly expressed in Pro-mac cells that promote tumor growth, angiogenesis and suppress tumor immunity (**Figure 6f** [↗](#)). We obtained the Pro-mac and Anti-mac cells from 12 cases (6 cases of surgery alone, 6 cases of surgical samples after neoadjuvant chemotherapy) by flow cytometry. We named them Control Anti-mac, Control Pro-mac, NCT Anti-mac, NCT Pro-mac. After placing them in a cell culture flask for 24 hours, the content of some key cytokines in the supernatant of the culture medium was detected by enzyme-linked immunosorbent assay (ELISA). The levels of MMP9, EGF, and VEGF secreted by Pro-mac after neoadjuvant chemotherapy were significantly higher than those of Pro-mac from the surgery alone group. MMP9, EGF, VEGF, and IL10 secreted by Pro-mac were significantly higher than Anti-mac (**Figure 6g** [↗](#)). Similarly, when Control Anti-mac, Control Pro-mac, NCT Anti-mac, and NCT Pro-mac were inoculated

subcutaneously with A549 cells at a ratio of 1:1 (Reinjection of macrophages two weeks later), we also found that NCT Pro-mac can significantly promote tumor growth. Interestingly, NCT Anti-mac in the tumor microenvironment after chemotherapy can significantly inhibit the growth of tumor cells, and this inhibitory ability was stronger than Control Anti-mac (**Figure 6h** [, i](#)).

Our previous analysis found that Pro-mac glycolysis-related signaling pathways were significantly enriched, while in Anti-mac, oxidative phosphorylation and fatty acid metabolism signaling pathways were greatly enhanced (**Figure 6j** [, k](#)). In vitro experiments show that NCT Pro-mac's ability to take up glucose and produce lactate was considerably more potent than other cells (**Figure 6k** [, l](#)). It was worth noting that the glycolysis level of NCT Anti-mac was markedly higher than that of Control Anti-mac (**Figure 6l** [, m](#)). When we placed the Pro-mac and Anti-mac in a 24-well plate and co-cultured with A549 cells in the Transwell chamber, we found that NCT Pro-mac can significantly enhance the invasion ability of A549 cells. At the same time, NCT Anti-mac showed a stronger ability to inhibit tumor invasion than Control Anti-mac (**Figure 6m** [, n](#)). However, when we used 2-DG (800uM, the concentration determined in pre-experiment) to inhibit the glycolysis of TAMs, the ability of Pro-mac to promote tumor progression was significantly weakened, and the power of NCT Anti-mac to suppress tumors was also considerably reduced (**Figure 6m** [, n](#)). By mixing these cells with macrophages for 3D culture, we found that the ability of NCT Anti-mac to inhibit tumor proliferation was significantly weakened when inhibiting its glycolytic activity. This showed that glycolysis could enhance the ability of Pro-mac to promote tumor progression and increase the capacity of Anti-mac to inhibit tumors (**Figure 6n** [, o](#)). Finally, through in vivo experiments, we inoculated a mixture of TAMs and A549 to nude mice and obtained the same experimental results as in **Figure 6m** [, n](#) (**Figure 6o** [, p](#)).

Chemotherapy treatment-induced remodeling of T and B cells

Considering the essential role of the tumor microenvironment, especially the immune infiltration level, in tumor development and response to therapy, we next investigated the characteristics of T and B cells. In our study, 22530 T cells were detected, which accounted for 26.9% of the total. We noticed that the re-clustered T cells could not be visibly distinguished among patients receiving different therapeutic regimens (Supplementary Figure 6a-b). According to the expression of a series of canonical markers of T cell subtypes, the T cells were divided into CD4⁺ T (marked by LTB, CD45RO, etc.), CD8⁺ T (marked by NKG7, GZMA, GZMB, CD8A, etc.), and Tregs (marked by FOXP3, CTLA4, etc.) ([12](#) [, 21](#) [, 29](#)) (Supplementary Figure 6c-d). The detailed expression profile of these marker genes is exhibited in Supplementary Figure 6e. Meanwhile, aside from these previously published T-cell markers, we also noted the specific upregulation of several genes in a particular cluster. At the same time, their expression specificity has not been elucidated yet.

As the major executor of tumor immunology, CD8⁺ T cells are thought to differentiate into cytotoxic T cells (CTLs) and specifically recognize endogenous antigenic peptides presented by the major histocompatibility complex I, thereby eliminating tumor cells ([30](#)). By comparing the composition of T cell subtypes in LUAD cells derived from different groups, we found that the proportion of CD8⁺ T cells in the NCT group was significantly higher than those in patients receiving only surgical treatment (Supplementary Figure 6d). Therefore, we focused on CD8⁺ T cells for subsequent analyses and re-clustered them into five new subgroups, in which clusters 1-4 were mainly derived from the NCT group. In contrast, cluster 5 was predominantly enriched in the control group (Supplementary Figure 6f-k).

We next explored the expression profile of genes associated with T cell's function in each CD8⁺ T sub-cluster. As depicted in Supplementary Figure 6i, clusters 1 and 2 were characterized by up-regulated naïve T cell markers, such as TCF7, LEF1, and CCR7, whereas genes associated with immune inhibition, like TIGIT, CTLA4, PDCD1, and HAVCR2, were enriched explicitly in cluster 3. Cytotoxic function-related genes, including GZMA GNLY, PRF1, GZMP, and GZMK, IFNG, IL2, were respectively overexpressed in clusters 4 and 5. Based on this evidence, we defined clusters 1 and 2 as naïve T, three as regulatory/exhausted T, and 4 & 5 as cytotoxic T cells. Intriguingly, regarding

both the sample origins and expression profiles of CD8⁺ T cells in clusters 4 and 5, we can reasonably hypothesize that NCT treatment potentially induces the reprogramming of CD8⁺ cytotoxic cells. To further verify this statement, we performed pseudotime-ordered trajectory analysis to monitor the dynamic view of CD8⁺ T cells' reprogramming process via Monocle. As shown in Supplementary Figure 6l-p, three phases were detected in these clusters. Cluster 1, which exhibited the lowest cytotoxicity, was designated as the "root" state according to pseudotime.

In contrast, the immune inhibition-related genes like LAG3, TIGIT, and PDCD1, and cytotoxicity-related genes such as GZMB and IFNG were respectively activated in phases 2 and 3. This phenomenon is consistent with our T cell phenotype classification mentioned above. Then, our results showed differentiation paths from naive T to Treg/exhausted cells and cytotoxic cells. Considering the transcriptional changes associated with T cell reprogramming, naive T cells (phase 1) expressing high CCR6 and TCF7 differentiate into two distinct fates, clusters 4 and 5, in phase 3. Notably, the cells positioned at the cluster 4 branch were characterized by higher cytotoxicity than in cluster 5 (Supplementary Figure 6l, m, o). Regarding the sample origins of the two clusters, these findings demonstrated that NCT treatment ignites a relatively more robust immune cytotoxic response towards tumor cells, which could be partly explained by the excessive production of neoantigen caused by NCT-induced DNA damage.

SCENIC analyses suggested that distinct transcriptional mechanisms drove the differentiation of naive T cells to either cluster 4 or 5. As revealed in Supplementary Figure 6q, the cytotoxic cells derived from NCT-treated LUAD patients (cluster 4) were characterized by increased activation of FOSL2-extended, REL, YBX1, and NF-KB pathways. In contrast, those from the control group (cluster 5) had up-regulated JUN, FOSB, and ELF3 extended pathways. Together, our results revealed that preoperative chemotherapy prompts the naive T cells to differentiate towards a more cytotoxic phenotype.

As for B cells, only 3902 (4.6%) cells were detected. 475 cells were derived from the control group, while 3427 were from the NCT group (Supplementary Figure 4a). Herein, we re-clustered the B cells into two sub-clusters. Based on canonical cell markers, class-switched memory B-cells (marked by CD19, CD37, and HLA-DRA) and plasma cells (marked by IGHA2, IGHG4, and CD38) were defined (Supplementary Figure 4a-c). The former comprised the majority of the total B cells (80.7%). Notably, the sample origins of the B cells demonstrated that a higher proportion of plasma cells characterized the control groups. In contrast, the class-switched memory B cells were significantly enriched in preoperatively treated patients.

Meanwhile, we performed GSVA analysis to explore several key biological pathways in the B cells derived from different groups. As depicted in Supplementary Figure 4d, B cells from the control group exhibited significant activating ways associated with metabolism and energy supply, including glycolysis and oxidative phosphorylation. However, the B cells derived from the NCT group exerted essential roles in most of the pathways, including glycolysis, fatty acid metabolism, apoptosis, and hypoxia. Overall, our observations demonstrated that NCT not only induced T cell reprogramming but also extensively impacted the composition and function of B cells in the tumor microenvironment.

Crosstalk among tumor and immune cells

The tumor microenvironment consists of numerous cell types, and the importance of crosstalk between cancer and immune cells has been implicated in various biological processes associated with tumor development (21, 29, 31). As depicted in Supplementary Figure 7a-b and Supplementary Figure 5a, the interactions between malignant cells and macrophages exhibited the strongest activity in both control and NCT groups, highlighting the important role of the macrophage in tumor immunology. Notably, we noted that the cell-to-cell communications among different cell types, especially between tumoral and immune cells such as cytotoxic CD8⁺ T, Treg, and memory B, were significantly strengthened in the NCT group. Specifically, we further

investigated the ligand-receptor atlas within and between tumor cells and immune cells, which seemed to be quite reshaped by NCT (Supplementary Figure 7c-d, Supplementary Figure 5b). For example, MIF-CXCR4, whose activation usually promotes leukocyte recruitment (32), was increasingly activated in the NCT group between malignant and memory B, CD4+ T, and cytotoxic CD8+ T, whereas inhibited in macrophages. Meanwhile, MDK-NCL exhibited a similar activating phenotype with MIF-CXCR4, but its function in shaping the tumor microenvironment has never been reported. So, it might serve as a potential target of immune checkpoint inhibitor treatment in the future.

Given the above-mentioned NCT-induced immune activation, which was characterized by CD8+ T with higher cytotoxicity and an increased proportion of class-switched memory B cells, these findings further clarified that NCT could ignite a strong intrinsic immune response towards tumor cells. However, the inhibitory interaction pairs LGALS9-CD44 and LGALS9-HAVCR2 was abnormally activated in the NCT group between malignant and several T cells or macrophages (33). Its exact role in such conditions still requires further exploration.

In summary, our study revealed that the LUAD tissues that have experienced NCT had a distinct landscape of intracellular interactions, which might provide new ideas for future research focusing on implementing immunotherapy in the comprehensive anti-tumor therapeutic regimen.

Discussion

Although important advances in chemotherapy have reduced the mortality of cancer patients, the 5-year survival rate is still low, mainly due to the inherent or acquired mechanism of anti-tumor drug resistance (34). Chemoresistance results from complex reprogramming processes, such as drug export/import, drug detoxification, DNA damage repair, and cell apoptosis. Recently, the correlation between metabolic regulation and chemoresistance has received great attention. More efforts are devoted to targeting cell metabolism to overcome chemoresistance (35). The classic mechanism is to target the transport of anti-cancer drugs by increasing the activity of the efflux pump, such as the adenosine triphosphate (ATP) binding cassette (ABC) transporter. Cancer cells exhibit a special metabolic phenotype-aerobic glycolysis, quickly transporting and consuming glucose to produce ATP and promote drug efflux. PI3K/AKT pathway is activated by producing 3'-phosphorylated phosphoinositol, which is an important signaling pathway for lung cancer MDR (36). Glycolysis is beneficial to cancer cells by producing ATP faster, providing many intermediates for violent biosynthesis, maintaining redox balance, and creating a microenvironment with low immunity (24). The combination therapy of shikonin+2-DG could inhibit glycolytic phenotype, migration, and invasion by regulating the Akt/HIF1 α /HK-2 signal axis (37).

Normal and healthy cells mainly produce energy through OXPHOS. However, due to rapid cell growth and frequent division, cancer cells face impressive metabolic challenges, which force them to adjust their energy metabolism to meet these needs (38). It is generally believed that cancer cells mainly obtain energy through glycolysis, which is named the Warburg effect. After chemotherapy, cancer cells change their metabolism from glycolysis to OXPHOS. This process is regulated by the SIRT1-PGC1 α signaling pathway, thus increasing the resistance of cells to chemotherapy (38). Drug-resistant cancer cells can often be re-sensitized to anti-cancer treatments by targeting the metabolic pathways of import, catabolism, and synthesis of basic cell components (40). Recent studies have determined the cancer-promoting function of mitochondrial oxidative phosphorylation (OXPHOS) by regulating cell growth and redox homeostasis (41). Our study also found that after chemotherapy, the glycolysis and oxidative phosphorylation of tumor cells was enhanced. This metabolic reprogramming may enable cancer cells to have higher proliferation, invasion, and metastasis capabilities.

Tumor endothelial cells (ECs) have high glycolytic metabolism, shunting intermediates to nucleotide synthesis. Blocking of the glycolysis activator PFKFB3 in EC cells does not affect tumor growth. Still, it reduces cancer cell invasion, intravascular, and metastasis by normalizing tumor blood vessels, thereby improving blood vessel maturation and perfusion. PFKFB3 inhibition tightens the vascular barrier by reducing VE-cadherin endocytosis in endothelial cells and reduces glycolysis to make cells more quiescent and adherent (by up-regulating N-cadherin); it also reduces NF- κ B signaling to reduce the expression of cancer cell adhesion molecules in ECs. PFKFB3 blockade therapy also improves chemotherapy for primary and metastatic tumors (42 [↗](#)).

Due to rapid cell growth and frequent division in tumor cells, cancer cells face impressive metabolic challenges, which force them to adjust energy metabolism to meet these needs, namely metabolic reprogramming (43 [↗](#)). However, studies have shown that metabolic plasticity in tumors is contributed by the glycolytic phenotype (as explained by Warburg) and that mitochondrial energy reprogramming has recently been identified as a feature of tumors (44 [↗](#)). Chemotherapy can increase SIRT1/PGC1 α -dependent oxidative phosphorylation (OXPHOS) in tumor cells, thereby promoting the survival of colorectal tumors during treatment. This phenomenon was also observed in chemotherapy-exposed liver metastases, which strongly suggests that chemotherapy causes long-term changes in tumor metabolism, which may interfere with drug efficacy (39 [↗](#)). In addition, elevated glycolysis and OXPHOS promote epithelial-mesenchymal transition and cancer stem cell (CSC) phenotype in tumor cells (45 [↗](#)). Therefore, recent research emphasizes the mixed glycolysis/OXPHOS phenotype rather than the phenotype that relies excessively on glycolysis to meet cellular energy requirements, thereby significantly promoting aggressiveness and treatment resistance (44 [↗](#)). Chemotherapy has a significant effect on the metabolic reprogramming of tumor cells and profoundly affects stromal and immune cells' metabolism in the tumor microenvironment.

Theoretically, chemical drugs can inhibit tumorigenesis by blocking the proliferation of tumor cells or depositing in tumor cell apoptosis, but this unintentionally causes "tissue damage." The body will mistake this tumor-specific damage for normal tissue damage and then inevitably activate the tissue damage repair mechanism dominated by TAM (46 [↗](#)). The result of this effect is that tumors will grow rapidly, and patients will develop resistance to anti-tumor chemotherapy.

Meanwhile, as suggested by Parra et al., neoadjuvant chemotherapy exerted PD-L1 upregulation in NSCLC patients. It increased the density of CD68+ macrophages, which were associated with better outcomes in both univariate and multivariate analyses (47 [↗](#)). However, opposite results were also reported by Talebian et al. that NSCLC patients treated with radiotherapy, rather than a platinum-based standard-of-care chemotherapy, displayed a decrease in lymphoid cells and a relative increase in macrophages (48 [↗](#)). Therefore, the role of TAMs in LUAD cells' response to chemotherapy still requires further investigation.

Our research reveals the remodeling effect of chemotherapy on tumor microenvironment. However, this study still has many limitations. Firstly, only 9 samples were included in our study, and the number of samples is a defect of our study. Secondly, our study did not detect other causes of tumor heterogeneity, such as EGFR-mutant or ALK-translocated. We explored the possible impact of these factors and found that there was no significant difference in the expression of EGFR and other genes between the NCT group and the Control group (Supplementary Table 1). Thirdly, our data can only reflect the change in gene expression of various types of cells in the tumor microenvironment after chemotherapy. We can not draw a direct conclusion on whether chemotherapy will benefit patients or not. These need further study in the future.

Availability of data and material

The single-cell sequencing data used in this study and meta data (including) can be obtained from the figshare (10.6084/m9.figshare.24797265).

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Additional information

Author contributions

S.Y.Y, Y.W.H., G.S.B, C.Z., and G.J.W. designed the research; Y.W.H., L.C., J.Q.L., T.L., M.N.Z., G.S.B., M.L., J.Q.L., Z.Y.H., Y.S.Z., J.J.X, C.Z., Z.W.L., W.J., Q.W., and L.J.T. performed the research; Y.W.H., G.J.W., C.Z., L.X., and G.S.B. analyzed the data; Y.W.H., G.J.W., G.S.B., J.Q.L., L.X., and C.Z. wrote the paper. Final approval of the manuscript: All authors.

Declarations

A total of 9 patients were included in this study. The Ethics Committee of Zhongshan Hospital of Fudan University approved our research (B2021-230R), and the content of the study complies with the Helsinki Declaration.

A total of 60 animals were involved in this study. They were treated humanely and received standard care. The animal experimental procedures were approved by the Institutional Review Board of Zhongshan Hospital of Fudan University (Shanghai, China).

Competing interests

The authors have no conflicts of interest to declare.

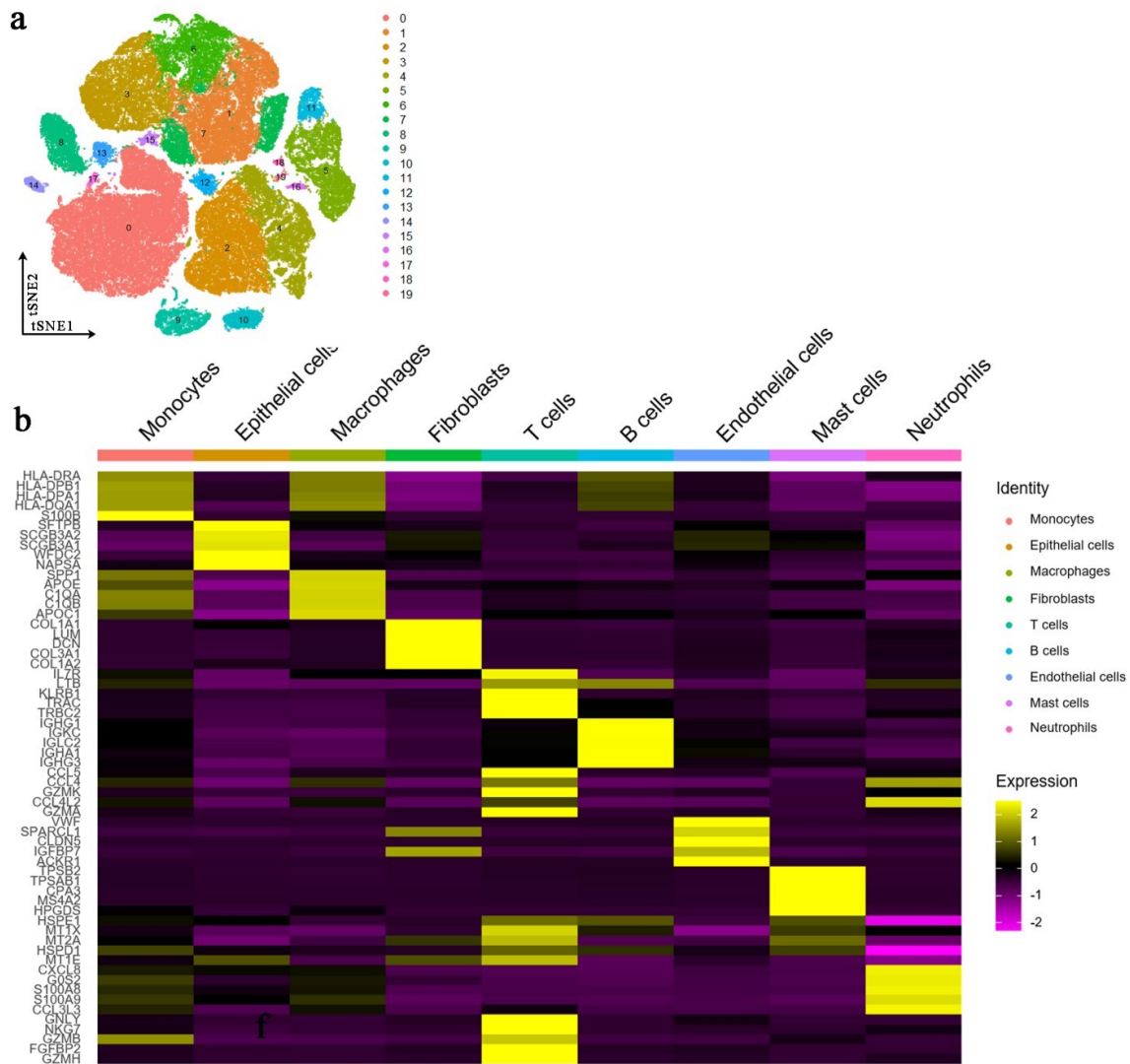
Consent for publication

All of the authors have consented to publication.

Funding

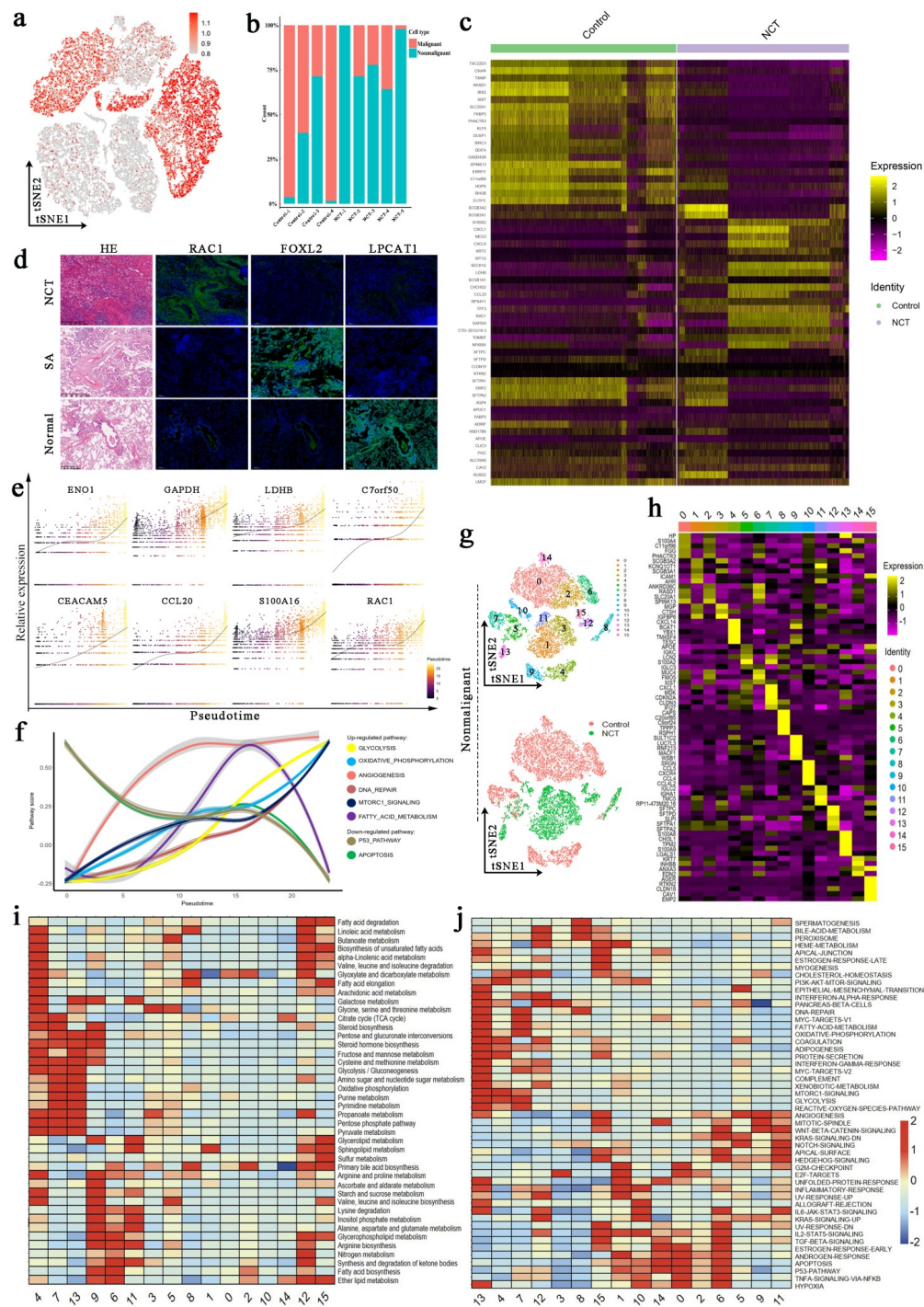
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Supplementary figures



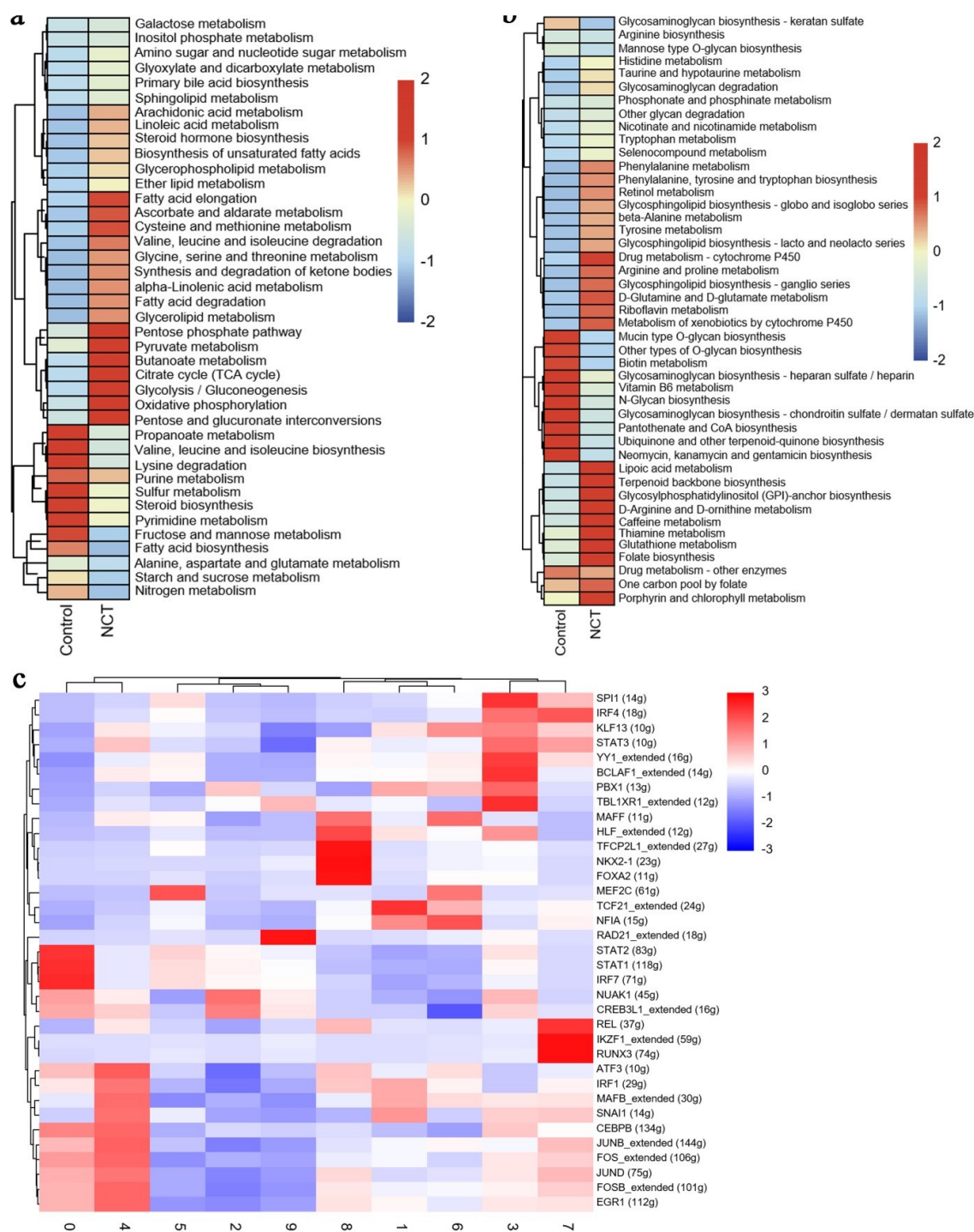
Supplementary Figure 1.

Figure related to **Figure 1**. **(a)** The tSNE plots of all clusters in this research. **(b)** The heatmap showed the marker genes in the different cell types.



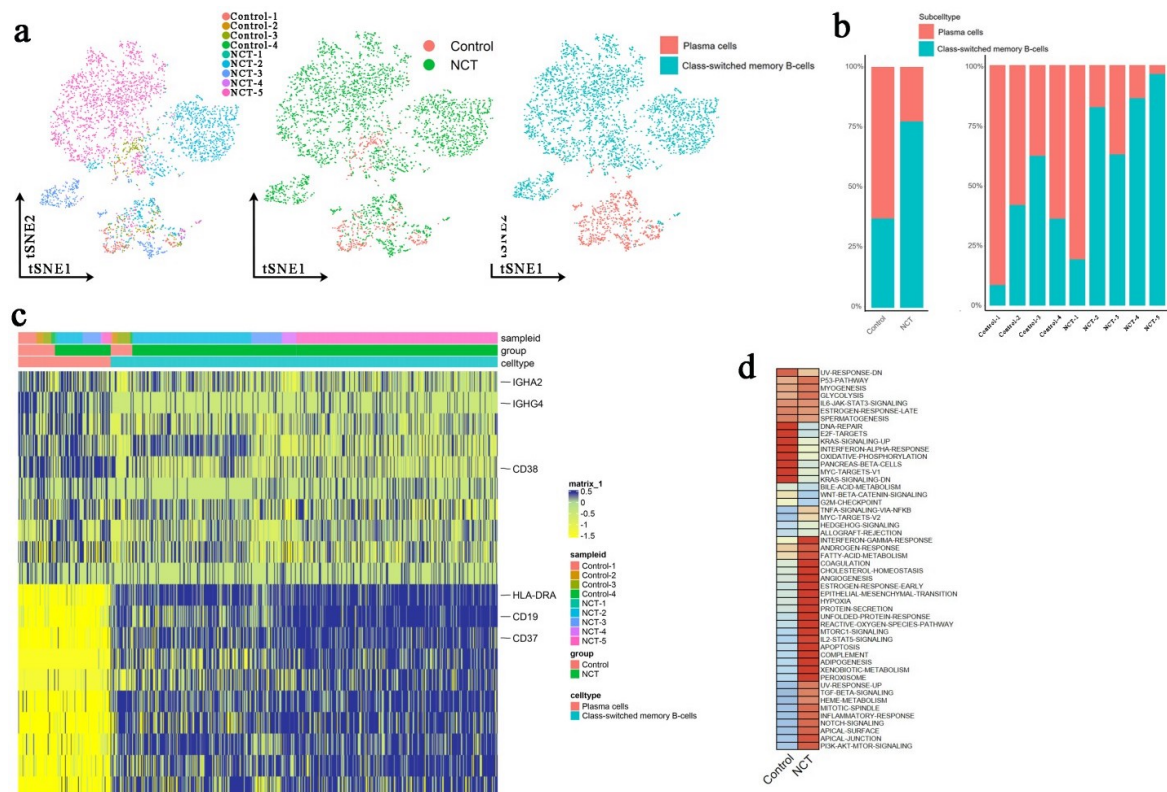
Supplementary Figure 2.

Figure related to **Figure 3**. **(a)** Copy number variations (CNVs) of malignant and nonmalignant epithelial cells. **(b)** The proportion of malignant cells and nonmalignant cells from different patients. **(c)** The heatmap showed differentially expressed genes of epithelial cells in the Control group and NCT group. **(d)** Immunofluorescence showed LPCAT1, FOXL2, and RAC1 were highly expressed in normal lung tissues, the Control group, and the NCT group, respectively. **(e)** Some genes played an important role in the transformation of epithelial cells into tumor cells. **(f)** Changes in the activity of several important pathways during the transformation of epithelial cells to tumor cells. **(g)** The tSNE plots and overview of the non-malignant cells. **(h)** Heat map of marker genes for nonmalignant cells sub-clusters. **(i)** Metabolic characteristics in different nonmalignant cell sub-clusters. **(j)** GSVA analysis revealed the characteristics of pathway activity in different nonmalignant cell sub-clusters.



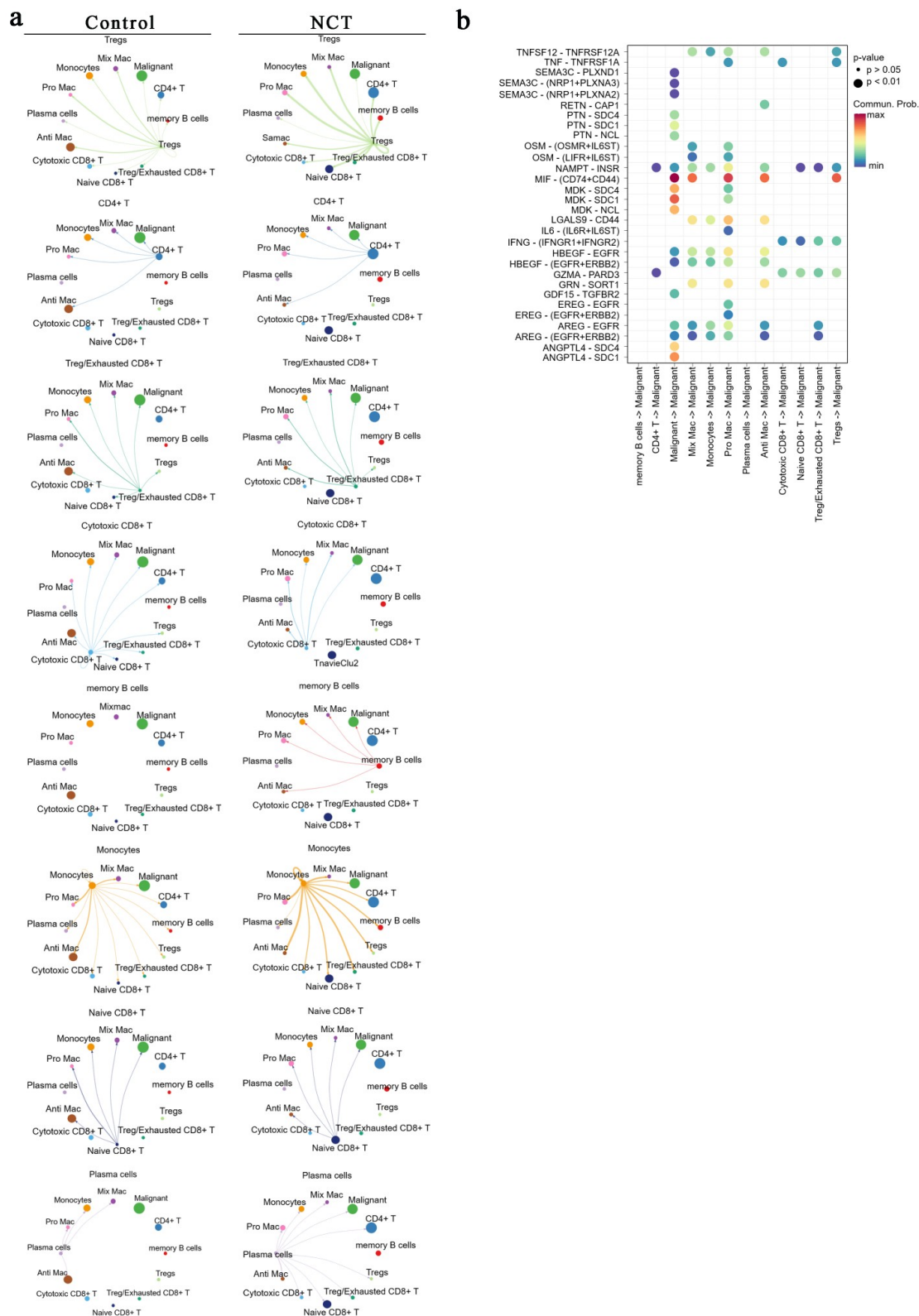
Supplementary Figure 3.

Figure related to [Figure 4](#). **(a)** The activity of various metabolic processes in fibroblasts from the Control group and NCT group. **(b)** GSEA analysis was performed for fibroblasts from the Control group and NCT group. **(c)** SCENIC analysis revealed the hub genes in fibroblast.



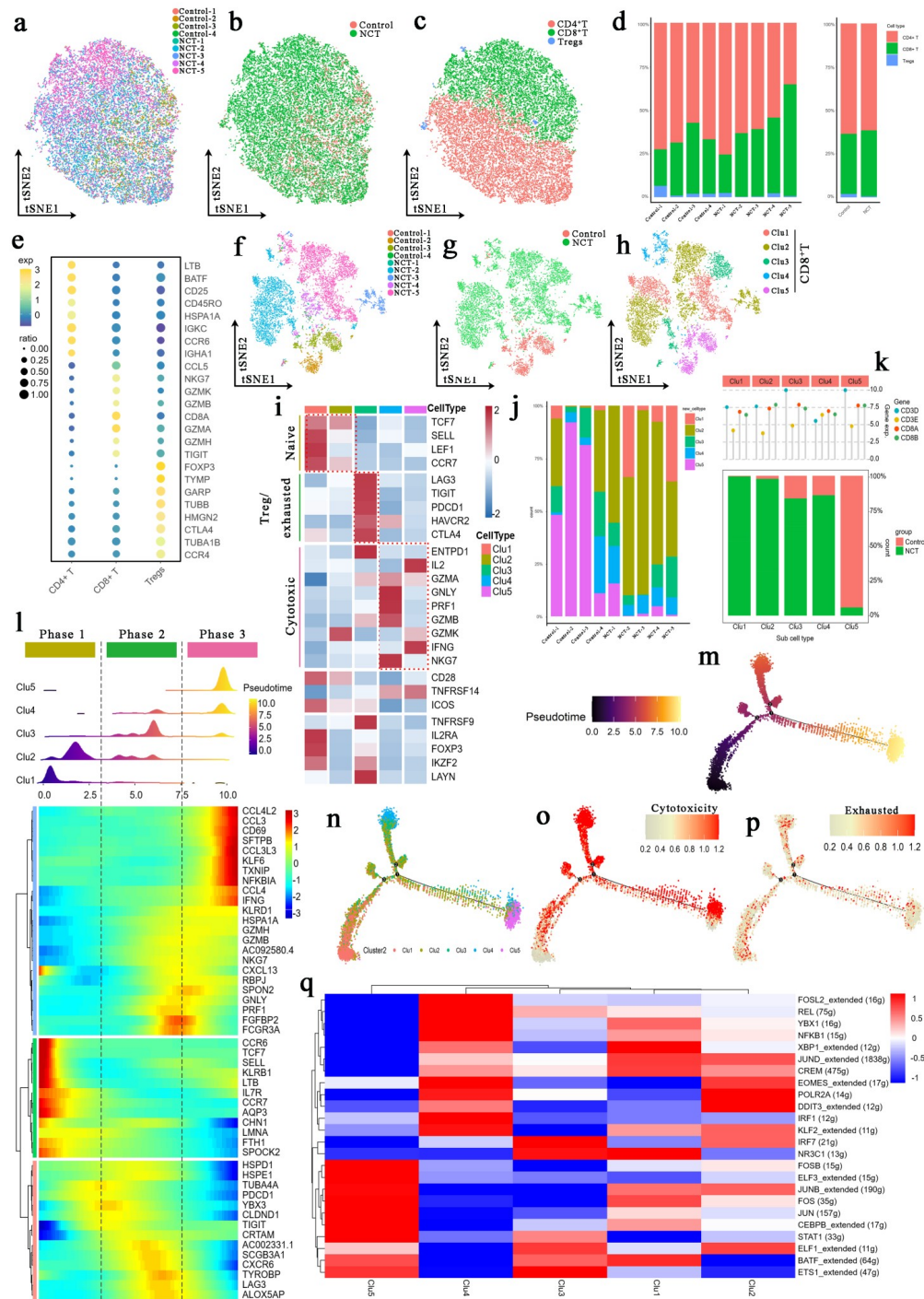
Supplementary Figure 4.

The scRNA profile of B cells derived from LUAD samples in the control, neoadjuvant chemotherapy, and immunotherapy group. **(a)** The tSNE plots revealed the sample origins, group origins, and predicted clusters of B cells. **(b)** The two predicted clusters of B cells (plasma cells, class-switched memory B-cells) were reported in different groups and samples. **(c)** Heatmap exhibiting the expression level of marker genes in each B cell cluster. **(d)** GSVA analysis estimated the pathway activation levels of different B cell subtypes.



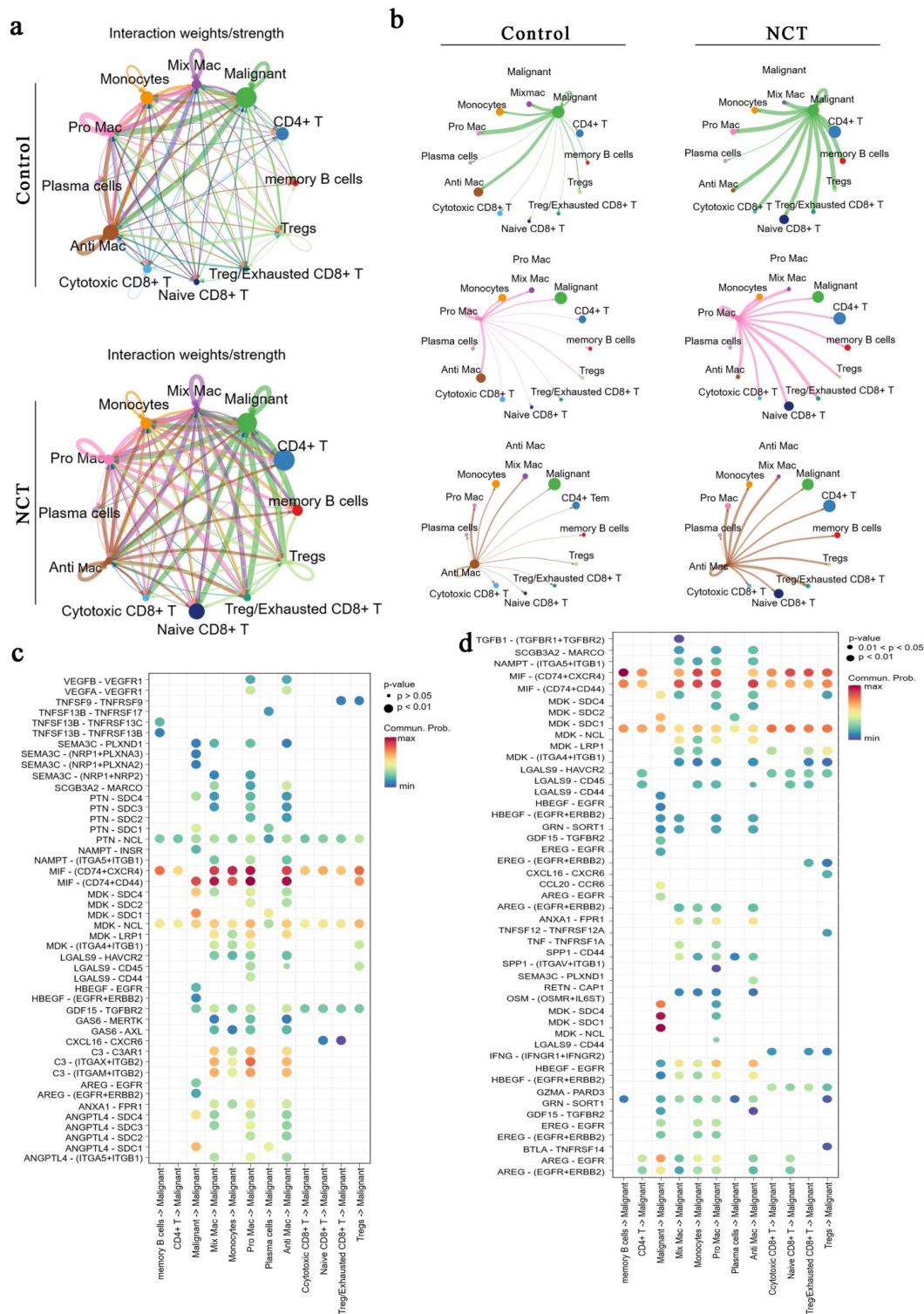
Supplementary Figure 5.

Crosstalk between cancer and immune cells. **(a)** Each cell type and the other cell types expressed some of the ligands. **(b)** Bubble plot revealing the specific ligand-receptor interactions between cancer cells and immune cells in the control group. The circle size indicates P values, with the scale to the right (permutation test), and color indicates communication probability.



Supplementary Figure 6.

The scRNA profile of T cells derived from LUAD samples in the control, neoadjuvant chemotherapy, and immunotherapy group. **(a-c)** The tSNE plots revealed the sample origins **(a)**, group origins **(b)**, and predicted clusters **(c)** of T cells. **(d)** The three indicated clusters of T cells (CD4+ T, CD8+ T, and Tregs) were reported in different groups and samples. **(e)** Bubble plot exhibiting the expression level of marker genes in each T cell cluster. **(f-h)** The tSNE plots revealed the sample origins **(f)**, group origins **(g)**, and predicted subclusters **(h)** of CD8+ T cells. **(i)** Heatmap exhibiting the expression level of marker genes corresponding to naïve, Treg/exhausted, and cytotoxic phenotypes in each CD8+ T cell subcluster. **(j-k)** Proportions of the five predicted clusters of CD8+ T cells in different samples **(j)** and groups **(k)**. **(l)** Dynamic changes in gene expression of CD8+ T cells during the transition (divided into three phases). **(m-p)** Pseudotime-ordered analysis of CD8+ T cells **(m-n)** revealing the dynamics of their cytotoxic **(o)** and exhausted levels **(p)**. **(l)** SCENIC analysis of CD8+ T cells.



Supplementary Figure 7.

Crosstalk between cancer and immune cells. **(a)** Overview of selected ligand-receptor interactions of cancer cells and immune cells in control and NCT groups. The line thickness indicates the number of ligands when cognate receptors are present in the recipient cell type. The loops indicate autocrine circuits. **(b)** Detailed view of the ligands expressed by each cell type and the other cell types. **(c)** Bubble plot revealing the specific ligand-receptor interactions between cancer cells and immune cells in the NCT group. The circle size indicates P values, with the scale to the right (permutation test), and color indicates communication probability.

Abbreviations

- LUAD: lung adenocarcinoma
- NCT: neoadjuvant chemotherapy
- TME: tumor microenvironment
- TSNE: t-distributed random neighborhood embedding
- CNV: copy number variation
- SCENIC: Single-Cell Regulatory Network Inference and Clustering
- SMC: smooth muscle cells
- TCA cycle: pyruvate metabolism and citrate cycle
- TAM: Tumor-Associated Macrophages
- CTLs: cytotoxic T cells
- OXPHOS: oxidative phosphorylation
- CSC: cancer stem cell
- GSVA: Gene Set Variation Analysis
- NMF: Non-negative Matrix Factorization

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Reviewer #1 (Public review):

Summary:

This study reports single-cell RNA sequencing results of lung adenocarcinoma, comparing 4 treatment-naïve and 5 post-neoadjuvant chemotherapy tumor samples.

The authors claim that there are metabolic reprogramming in tumor cells as well as stromal and immune cells after chemotherapy.

The most significant findings are in the macrophages that there are more pro-tumorigenic cells after chemotherapy, i.e. CD45+CD11b+ARG+ cells. In the treatment-naïve samples, more anti-tumorigenic CD45+CD11b+CD86+ macrophages are found. They sorted each population and performed functional analyses.

Strengths:

Comparison of the treatment-naïve and post-chemotherapy samples of lung adenocarcinoma.

Weaknesses:

After the revision, issues remain with lengthy descriptive clustering type analysis, insufficient statistical support, and inefficient figure presentation.

<https://doi.org/10.7554/eLife.95988.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

This study reports single-cell RNA sequencing results of lung adenocarcinoma, comparing 4 treatment-naïve and 5 post-neoadjuvant chemotherapy tumor samples. The authors claim that there are metabolic reprogramming in tumor cells as well as stromal and immune cells after chemotherapy.

The most significant findings are in the macrophages that there are more pro-tumorigenic cells after chemotherapy, i.e. CD45+CD11b+ARG+ cells. In the treatment-naïve samples, more anti-tumorigenic CD45+CD11b+CD86+ macrophages are found. They sorted each population and performed functional analyses.

Strengths:

Comparison of the treatment-naïve and post-chemotherapy samples of lung adenocarcinoma.

Weaknesses:

(1) Lengthy descriptive clustering analysis, with indistinct direct comparisons between the treatment-naïve and the post-chemotherapy samples.

Thank you for your detailed review and valuable feedback. We have simplified the descriptive clustering analysis by removing redundant parts and retaining only the key content relevant to our findings. This should help readers to more easily grasp and focus on the main results.

(2) No statistical analysis was performed for the comparison.

We appreciate your constructive feedback and are committed to improving our research methodology and reporting to enhance the scientific rigor of our studies.

(3) Difficult to match data to the text.

Thank you for your feedback. We understand that there were difficulties in matching the data to the text. We have reviewed the manuscript carefully to ensure that all data points are clearly linked to the corresponding sections in the text.

(4) ARG1 is a cytosolic enzyme that can be detected by intracellular staining after fixation. It is unclear how the staining and sorting was performed to measure function of sorted cells.

We apologize for the error caused by miscommunication within our research team. We are currently using both ARG1 and CD206 antibodies in our studies. Due to a communication error, the technician mistakenly assumed ARG1 was another name for CD206 (MRC1), resulting in the incorrect labeling of CD206 as ARG1 in our experimental records. In reality, we used the CD206 antibody, which is consistent with the same surface marker shown in figure 6e. We have made corrections in the manuscript and experimental figures. Thank you for pointing this out, and we regret any misunderstanding this may have caused.

Reviewer #2 (Public Review):

In this study, Huang et al. performed a scRNA-seq analysis of lung adenocarcinoma (LUAD) specimens from 9 human patients, including 5 who received neoadjuvant

chemotherapy (NCT), and 4 without treatment (control). The new data was produced using $10 \times$ Genomics technology and comprises 83622 cells, of which 50055 and 33567 cells were derived from the NCT and control groups, respectively. Data was processed via R Seurat package, and various downstream analyses were conducted, including CNV, GSVA, functional enrichment, cell-cell interaction, and pseudotime trajectory analyses. Additionally, the authors performed several experiments for in vitro and in vivo validation of their findings, such as immunohistochemistry, immunofluorescence, flow cytometry, and animal experiments.

The study extensively discusses the heterogeneity of cell populations in LUAD, comparing the samples with and without chemotherapy. However, there are several shortcomings that diminish the quality of this paper:

- The number of cells included in the dataset is limited, and the number of patients from different groups is low, which may reduce the attractiveness of the dataset for other researchers to reuse. Additionally, there is no metadata on patients' clinical characteristics, such as age, sex, history of smoking, etc., which would be valuable for future studies.

Thank you for your insightful feedback. We recognize that the limited number of cells and the small number of patients from different groups in our dataset may affect its appeal for reuse by other researchers. Additionally, we acknowledge the absence of metadata on patients' clinical characteristics, such as age, sex, and smoking history, which would indeed be valuable for future studies. We have compiled statistics on the patient's metadata and other information in the Supplementary Table 2.

We appreciate your suggestions and will consider incorporating these aspects in future research to enhance the dataset's utility and attractiveness.

- Several crucial details about the data analysis are missing: How many PCs were used for reduction? Which versions of Seurat/inferCNV/other packages were used? Why monocle2 was used and not monocle3 or other packages? Also, the authors use R version 3.6.1, and the current version is 4.3.2.

Thank you for your detailed review and valuable suggestions. Below are our responses to the points you raised:

Principal Components (PCs) Used for Reduction: We used the first 20 principal components (PCs) for dimensionality reduction. This choice was based on preliminary tests showing that 20 PCs captured the major variation in our data effectively.

Versions of Packages: The versions of the packages used are as follows:

Seurat: Version 4.0.1

inferCNV: Version 1.18.1

monocle2: Version 2.14.0

Choice of monocle2 over monocle3 or Other Packages: We chose monocle2 because it performed better on our specific dataset, and its algorithms suited our research needs. Additionally, we are more familiar with the functionalities and outputs of monocle2, which allowed us to better interpret and apply the results.

R Version: We used R version 3.6.1 at the beginning of our study to ensure consistency and reproducibility throughout the analysis. Although the current version of R is 4.3.2, we

maintained the same version throughout our research. We will consider upgrading to the latest version of R and re-testing for compatibility and performance in future studies.

We appreciate your attention to these details and will include this information in the revised manuscript.

• It seems that the authors may lack a fundamental understanding of scRNA-seq data processing and the functions of Seurat. For instance, they state, 'Next, we classified cell types through dimensional reduction and unsupervised clustering via the Seurat package.' However, dimensional reduction and unsupervised clustering are not methods for cell classification. Typically, cell types are classified using marker genes or other established methods.

Thank you for your insightful comments. We appreciate your guidance on the proper understanding and application of scRNA-seq data processing and the functions of Seurat.

You are correct in noting that dimensional reduction and unsupervised clustering are not methods for cell classification. We apologize for the confusion in our original statement. What we intended to convey was that we performed dimensional reduction and unsupervised clustering using the Seurat package as preliminary steps in our analysis. Following these steps, we classified cell types based on established marker genes.

"Therefore, to identify subclusters within each of these nine major cell types, we performed principal component analysis" (Line 127). Principal component analysis is a method for dimensionality reduction, not cell clustering.

The authors did not mention the normalization or scaling of the data, which are crucial steps in scRNA-seq data preprocessing.

Thank you for your insightful comments. We apologize for any confusion caused by our description in the manuscript. You are correct that principal component analysis (PCA) is primarily a method for dimensionality reduction rather than cell clustering. To clarify, we used PCA to reduce the dimensionality of our single-cell RNA-seq (scRNA-seq) data, which is a preliminary step before clustering the cells.

In the revised manuscript, we have provided a more detailed description of our data preprocessing pipeline, including the normalization and scaling steps that are indeed crucial for scRNA-seq data analysis. Specifically, we performed the following steps:

Normalization: We normalized the gene expression data to account for differences in sequencing depth and other technical variations.

Scaling: We scaled the normalized data to ensure that each gene contributes equally to the PCA, which mitigates the effect of highly variable genes dominating the analysis.

Following these preprocessing steps, we conducted PCA to reduce the dimensionality of the data, which facilitated the subsequent clustering of cells into subclusters.

We hope this addresses your concerns, and we appreciate your valuable feedback that helped us improve the clarity and accuracy of our manuscript.

• Numerous style and grammar mistakes are present in the main text. For instance, certain sections of the methods are written in the present tense, suggesting that parts of a protocol were copied without text editing. Furthermore, some sections of the introduction are written in the past tense when the present tense would be more suitable. Clusters are inconsistently referred to by numbers or cell types, leading to confusion. Additionally, the authors frequently use the term "evolution" when describing

trajectory analysis, which may not be appropriate. Overall, significant revisions to the main text are required.

Thank you for your detailed review and valuable feedback on our manuscript. We highly appreciate your suggestions and have made the following revisions to address the issues you pointed out:

Tense Consistency: We have thoroughly reviewed and corrected the use of tenses throughout the manuscript. The Methods section now consistently uses the past tense, while the Introduction section uses the present tense where appropriate, ensuring coherence and consistency.

Cluster Naming Consistency: We have standardized the naming conventions for clusters, consistently using either numbers or cell types to avoid any confusion.

Appropriate Terminology: We have reviewed our use of the term "evolution" in the context of trajectory analysis. Where necessary, we have replaced it with more accurate terms such as "trajectory progression" or "developmental pathway" to better convey the intended meaning.

• Some figures are not mentioned in order or are not referenced in the text at all, such as Figure 5l (where it is also unclear how the authors selected the root cells). Additionally, many figures have text that is too small to be read without zooming in. Overall, the quality of the figures is inconsistent and sometimes very poor.

Thank you for your detailed review and valuable feedback on our manuscript. We have addressed the issues you raised as follows:

Unreferenced Figures in the Text:

We acknowledge the oversight regarding Figure 5l not being mentioned in the text. In the revised version, we will ensure that all figures are properly referenced and discussed within the relevant sections of the manuscript.

Text Size in Figures:

We understand the difficulty in reading small text within the figures. We will redesign all figures to ensure that text and annotations are legible at normal viewing sizes. This will involve increasing the resolution and text size in all figures to enhance readability.

Inconsistent Quality of Figures:

To address the inconsistency in figure quality, we will standardize the formatting of all figures and ensure they meet a high standard of clarity and presentation. This will improve the overall visual quality and professionalism of the manuscript.

The results section lacks clarity on several points:

• The authors state that "myofibroblasts exclusively originated from the control group". However, pathways up-regulated in myofibroblasts (such as glycolysis) were enhanced after chemotherapy, as indicated by GSEA score. Similarly, why are some clusters of TAMs from the control group associated with pathways enriched in chemotherapy group?

Thank you for your insightful comments and questions regarding our manuscript. We appreciate the opportunity to clarify these points.

Regarding the statement that "myofibroblasts exclusively originated from the control group," we acknowledge the confusion and would like to provide a more detailed explanation. While the initial identification indicated that myofibroblasts were predominantly found in the

control group, subsequent analyses, including the Gene Set Variation Analysis (GSVA), revealed that certain pathways up-regulated in myofibroblasts, such as glycolysis, were indeed enhanced following chemotherapy. This suggests that chemotherapy may induce or enhance specific functional states in these cells that are not initially apparent from their origin alone.

Similarly, the observation that some clusters of Tumor-Associated Macrophages (TAMs) from the control group are associated with pathways enriched in the chemotherapy group can be explained by the dynamic nature of cellular responses to treatment. TAMs, like other immune cells, can exhibit plasticity and adapt to the tumor microenvironment altered by chemotherapy. This plasticity may result in the activation of pathways typically associated with a chemotherapy response, even in cells originating from the control group.

We will revise the manuscript to better articulate these findings and include additional data to support our explanations. This will help clarify the observed discrepancies and provide a more comprehensive understanding of the cellular dynamics in response to chemotherapy.

• Further explanation is necessary regarding the distinctions between malignant and non-malignant cells, as well as regarding the upregulation of metabolism-related pathways in fibroblasts from the NCT group. Additionally, clarification is needed regarding why certain TAMs from the control group are associated with pathways enriched in the chemotherapy group.

Thank you for your detailed review and for highlighting the areas that require further clarification. We appreciate the opportunity to provide additional explanations and improve our manuscript.

We recognize the need to more clearly differentiate between malignant and non-malignant cells in our manuscript. We will include additional details on the criteria and markers used to distinguish these cell types. Specifically, we will elaborate on the molecular and phenotypic characteristics that were used to identify malignant cells, such as specific genetic mutations, aberrant signaling pathways, and distinct cell surface markers, as opposed to those used for identifying non-malignant cells.

As mentioned above, the association of certain TAMs from the control group with pathways enriched in the chemotherapy group can be attributed to the inherent plasticity and adaptability of TAMs. We will provide a more detailed explanation of how TAMs can exhibit different functional states based on microenvironmental cues. This will include a discussion on the potential pre-existing heterogeneity within TAM populations and how even in the absence of direct chemotherapy exposure, some TAMs may display pathway activities similar to those seen in the chemotherapy group due to microenvironmental influences or intrinsic properties.

• In the section titled 'Chemo-driven Pro-mac and Anti-mac Metabolic Reprogramming Exerted Diametrically Opposite Effects on Tumor Cells': The markers selected to characterize the anti- and pro-macrophages are commonly employed for describing M1 or M2 polarization. It is uncertain whether this new classification into anti- and pro-macrophages is necessary. Additionally, it should be noted that pro-macrophages are anti-inflammatory, while anti-macrophages are pro-inflammatory, which could lead to confusion. M2 macrophages are already recognized for their role in stimulating tumor relapse after chemotherapy.

Thank you for your feedback. We appreciate the opportunity to clarify the rationale behind our terminology and the focus on functional phenotypic changes in macrophages before and after chemotherapy.

Our intention in introducing the terms "pro-macrophages" and "anti-macrophages" was to highlight the distinct functional phenotypic changes in macrophages observed before and after chemotherapy. These terms were chosen to emphasize the functional roles these macrophages play in the tumor microenvironment in response to chemotherapy, rather than strictly adhering to the conventional M1/M2 polarization paradigm.

We acknowledge that M2 macrophages are well-documented in stimulating tumor relapse after chemotherapy. Our use of "pro-macrophages" is intended to build on this established knowledge by providing a more nuanced understanding of their role in the post-chemotherapy tumor microenvironment. Similarly, "anti-macrophages" highlight the macrophages' role in mounting an anti-tumor response.

• The authors suggest that there is "reprogramming of CD8+ cytotoxic cells" following chemotherapy (Line 409). It remains unclear whether they imply the reprogramming of other CD8+ T cells into cytotoxic cells. While it is indicated that cytotoxic cells from the control group differ from those in the NCT group and that NCT cytotoxic T cells exhibit higher cytotoxicity, the authors did not assess the expression of NK and NK-like T cell markers (aside from NKG7), which may possess greater cytotoxic potential than CD8+ cytotoxic cells. This could also elucidate why cytotoxic cells from the NCT and control groups are positioned on separate branches in trajectory analysis. Overall, with 22.5k T cells in the dataset, only 3 subtypes were identified, suggesting a need for improved cell annotations by the authors.

Thank you for your valuable feedback regarding the classification and characterization of CD8+ cytotoxic cells following chemotherapy, and the need for improved cell annotations.

We appreciate your point on the potential ambiguity around the "reprogramming of CD8+ cytotoxic cells" post-chemotherapy. In our study, we observed that CD8+ T cells from the control and NCT groups differ significantly in their cytotoxic profiles, with the NCT group's cytotoxic T cells displaying enhanced cytotoxicity. However, we did not imply the reprogramming of other CD8+ T cells into cytotoxic cells. Instead, our findings suggest a shift in the functional state of existing CD8+ cytotoxic cells, driven by chemotherapy, which aligns with the upregulation of genes associated with cytotoxic functions.

We acknowledge that the expression of NK and NK-like T cell markers (apart from NKG7) was not comprehensively assessed. We agree that these markers may possess greater cytotoxic potential and could elucidate the separation observed in the trajectory analysis between cytotoxic cells from the NCT and control groups. This distinction may be attributed to differential cytotoxic potentials and functional states induced by chemotherapy.

Furthermore, with 22,530 T cells in the dataset, only three subtypes were initially identified. We recognize the need for more refined cell annotations to capture the full spectrum of T cell diversity. This could involve a deeper analysis of additional markers to distinguish between various cytotoxic populations, including NK and NK-like T cells, and their respective roles in the tumor microenvironment post-chemotherapy.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

I would recommend simplifying the manuscript and focusing on the differences between the treatment-naïve and post-chemotherapy samples.

Thank you for your valuable feedback on our manuscript. We greatly appreciate your suggestions and have carefully considered the proposed modifications.

Upon re-evaluating our manuscript, we believe that the current structure and content most effectively convey our research findings. Our study aims to not only compare the treatment-naïve and post-chemotherapy samples but also to highlight several important secondary findings that are integral to the overall research.

Nevertheless, we understand your recommendation to simplify the manuscript. To address this, we have made some subtle adjustments to improve the readability and conciseness of the text. Additionally, we have included a section in the discussion that more explicitly highlights the differences between the treatment-naïve and post-chemotherapy samples.

IRB number for the human sample collection as well as animal experiments need to be provided.

Thank you for your thorough review and for highlighting the need for the inclusion of the IRB number for the human sample collection and animal experiments.

We apologize for this oversight and appreciate your attention to this important detail. The Institutional Review Board (IRB) approval number for the human sample collection is [B2019-436].

This number has been added to the Methods section of our revised manuscript to ensure compliance with ethical standards and to provide transparency for our research.

I put a question on the macrophage sorting experiment in the public review. Please clarify how the ARG1 staining was achieved with the preservation of cell viability.

We apologize for the error caused by miscommunication within our research team. We are currently using both ARG1 and CD206 antibodies in our studies. Due to a communication error, the technician mistakenly assumed ARG1 was another name for CD206 (MRC1), resulting in the incorrect labeling of CD206 as ARG1 in our experimental records. In reality, we used the CD206 antibody, which is consistent with the same surface marker shown in figure 6e. We have made corrections in the manuscript and experimental figures. Thank you for pointing this out, and we regret any misunderstanding this may have caused.

Reviewer #2 (Recommendations For The Authors):

Minor comments:

• Line 65- "Chemotherapy drugs, however, are very toxic and are prone to invalid". Line 75-77: "This heterogeneity in the TME includes the differences between tumor cells and tumor cells and the differences between various stromal cells and immune cells. Actively exploring the changes of multiple cells in the TME of LUAD after chemotherapy may finally find an excellent way to overcome chemotherapy resistance for LUAD." Please rewrite these parts.

Thank you for your valuable comment. We have revised the manuscript according to your suggestion:

Original (Line 65): "Chemotherapy drugs, however, are very toxic and are prone to invalid."
Revised: "However, chemotherapy drugs are highly toxic and can often become ineffective."

Original (Line 75-77): "This heterogeneity in the TME includes the differences between tumor cells and tumor cells and the differences between various stromal cells and immune cells. Actively exploring the changes of multiple cells in the TME of LUAD after chemotherapy may finally find an excellent way to overcome chemotherapy resistance for LUAD."

Revised: "The heterogeneity within the tumor microenvironment (TME) encompasses not only the variations between different tumor cells but also among various stromal and immune cell types. Investigating the dynamic changes in multiple cell populations within the TME of LUAD following chemotherapy may provide crucial insights into overcoming chemotherapy resistance in LUAD."

• Line 87: *"The internal processes of the cells respectively drive immune cells and cancer cells to obtain glucose and glutamine preferentially."-> The internal metabolic changes in the cells drive...*

Thank you for your valuable comment. We have revised the manuscript according to your suggestion:

Original (Line 87): "The internal processes of the cells respectively drive immune cells and cancer cells to obtain glucose and glutamine preferentially."

Revised: "The internal metabolic changes in the cells drive immune cells and cancer cells to preferentially obtain glucose and glutamine."

• Line 93: *"an essential feature that affects the effect of chemotherapy"-> an essential feature that affects chemotherapy.*

Thank you for your valuable comment. We have revised the manuscript according to your suggestion:

Original (Line 93): "Metabolic reprogramming in various cell types in the tumor microenvironment after undergoing chemotherapy may be an essential feature that affects the effect of chemotherapy."

Revised: "Metabolic reprogramming in various cell types in the tumor microenvironment after undergoing chemotherapy may be an essential feature that affects chemotherapy."

• Line 84: *What do the immune cells depend on glucose for?*

Thank you for your valuable comment. We have revised the manuscript according to your suggestion:

Original (Line 84): "However, recent studies have shown that tumor-infiltrating immune cells depend on glucose and immune cells especially macrophages consume more glucose than malignant cells."

Revised: "However, recent studies have shown that tumor-infiltrating immune cells rely on glucose for their energy needs and functionality, with immune cells, particularly macrophages, consuming more glucose than malignant cells."

• Line 223: *"According to previous research, myofibroblast has been described"-> myofibroblasts have been described.*

Thank you for your valuable comment. We have revised the manuscript according to your suggestion:

Original (Line 223): "According to previous research, myofibroblast has been described as a cancer-associated fibroblast that participated in extensive tissue remodeling, angiogenesis, and tumor progression."

Revised: "According to previous research, myofibroblasts have been described as cancer-associated fibroblasts that participate in extensive tissue remodeling, angiogenesis, and tumor progression."

• Line 239: "*Considering the essential fibroblasts*"-> *Considering the essential role of fibroblasts.*

Thank you for your valuable comment. We have revised the manuscript according to your suggestion:

Original (Line 239): "Considering the essential fibroblasts and their complicated function in shaping the tumor microenvironment..."

Revised: "Considering the essential role of fibroblasts and their complicated function in shaping the tumor microenvironment..."

• Line 251: "*Further in vitro studies were required to elucidate these notable fibroblasts' potential function...*" -> *are required.*

Thank you for your valuable comments. We have revised the manuscript according to your suggestions:

Original (Line 251): "Further in vitro studies were required to elucidate these notable fibroblasts' potential function..."

Revised: "Further in vitro studies are required to elucidate these notable fibroblasts' potential function..."

• Line 309: "*Interestingly, we found that two subtypes, Anti-mac and Mix, can be converted to Pro-mac through pseudotime time analysis.*" -> *via trajectory analysis we found that two subtypes...*

Thank you for your valuable comments. We have revised the manuscript according to your suggestions:

Original (Line 309): "Interestingly, we found that two subtypes, Anti-mac and Mix, can be converted to Pro-mac through pseudotime time analysis."

Revised: "Interestingly, via trajectory analysis we found that two subtypes, Anti-mac and Mix, can be converted to Pro-mac."

• Line 458: "*the interactions between malignant and macrophages*"-> *the interactions between malignant cells and macrophages.*

Thank you for your valuable comments. We have revised the manuscript according to your suggestions:

Original (Line 458): "the interactions between malignant and macrophages"

Revised: "the interactions between malignant cells and macrophages."

• Line 486: "*The 5-year survival rate is still gloomy*" -> *The 5-year survival rate is still low.*

Thank you for your valuable comments. We have revised the manuscript according to your suggestions:

Original (Line 486): "The 5-year survival rate is still gloomy."

Revised: "The 5-year survival rate is still low."

• Line 491: *"More and more efforts are devoted to targeted metabolism to overcome chemoresistance" -> More efforts are devoted to target cell metabolism...*

Thank you for your valuable comments. We have revised the manuscript according to your suggestions:

Original (Line 491): "More and more efforts are devoted to targeted metabolism to overcome chemoresistance."

Revised: "More efforts are devoted to targeting cell metabolism to overcome chemoresistance."

• Line 594: *"Repeat the above steps twice" -> This procedure was repeated twice.*

Thank you for your valuable comments. We have revised the manuscript according to your suggestions:

Original (Line 594): "Repeat the above steps twice."

Revised: "This procedure was repeated twice."

• Line 620: *How were the new potential markers verified? List the exact genes and experiments or a reference to a Figure.*

Thank you for your valuable comments. We have provided detailed information on how the new potential markers were verified, including the exact genes involved and the specific experiments conducted. A reference to the relevant Figure has also been added to the manuscript.

• Line 637: *Which immune cells were used as a background in CNV analysis? All immune cells or just T cells?*

Thank you for your valuable comments. In this study, all immune cells were used as background control cells.

• Line 658: *in a single cell*

Thank you for your valuable comments. We have revised the manuscript according to your suggestions.

• Line 672: *"a variety of environmental factors potentially affect" -> potentially affects/ may potentially affect.*

Thank you for your valuable comments. We have revised the manuscript according to your suggestions:

Original (Line 672): "a variety of environmental factors potentially affect"

Revised: "A variety of environmental factors may potentially affect"

• Line 683: *Which metabolites were tested?*

The metabolites tested included those related to glycolysis and oxidative phosphorylation (OXPHOS), such as glucose and various metabolites indicative of mitochondrial activity. The contents of these metabolites were analyzed to verify consistency with gene expression levels as mentioned in the analysis of metabolic pathways section.

• *Line 718: Required or acquired?*

The correct term should be "acquired" in the context of discussing drug resistance in tumor cells. The sentence likely refers to the "acquired drug resistance" of tumor cells, which is a common challenge in chemotherapy.

• *Line 726: What are the A549 cells?*

A549 cells are a human lung adenocarcinoma cell line commonly used in cancer research, particularly for studying lung cancer. In this study, A549 cells were used in animal experiments, mixed with tumor-associated macrophages (TAMs), and implanted into nude mice to study tumor formation and progression.

• *Line 631: "we set the following cut-off thresholds to reveal the marker genes of each cluster: adjusted P-value <0.01 and multiple changes >0.5." What metric is "multiple changes"? Commonly used measures are adjusted P-value and average Log2FC.*

Thank you for your valuable comment. We have revised the manuscript according to your suggestion. The term "multiple changes" was indeed a misstatement. The correct metric should be "log2 fold change (Log2FC)," which is a commonly used measure in gene expression studies. We have updated the manuscript to reflect this, using "adjusted P-value <0.01 and average Log2FC > 0.5" instead of "multiple changes > 0.5."

• *Figure 1f: "Sampled" -> Samples. What do the numbers on the left side of each column mean?*

Thank you for your valuable comment. The term "Sampled" was indeed a typographical error and has been corrected to "Samples". The numbers on the left side of each column likely represent cluster IDs or sample identifiers corresponding to the different patient samples or clusters analyzed in the study. We have clearly labeled these numbers in the figure to avoid any confusion.

• *Figure 2b: Please add a scale.*

Thank you for your valuable comment. We agree that adding a scale bar is crucial for accurately interpreting the size of the cells or structures shown in the figure. We have now included an appropriate scale bar during the figure preparation stage to provide this reference.

• *Figure 3d/4c: What is the matrix_27/3 metric? Is it average expression?*

Thank you for your valuable comment. The term "matrix_27/3" refers to a specific metric used in our analysis. This metric indeed represents the average expression levels of genes within a particular subset of the dataset. We will clarify this in the figure legend and the methods section to ensure that readers have a clear understanding of what the metric represents. Additionally, we will make sure that all such metrics are consistently and accurately described throughout the manuscript.

- *Figure 6e: Why CD206 staining is shown instead of ARG if ARG was chosen as the main gene for classification of Pro-macrophages?*

We apologize for the confusion regarding the use of CD206 staining in Figure 6e. This issue arose due to a miscommunication within our research team. While ARG1 was initially intended as the primary marker for Pro-macrophages, the technician mistakenly assumed ARG1 was another name for CD206 (MRC1), leading to the incorrect labeling of CD206 as ARG1 in our experimental records. In actuality, CD206 was used for the staining, which is consistent with the surface marker shown in Figure 6e. We have corrected this error in the manuscript and updated the experimental figures accordingly. We sincerely apologize for any misunderstanding this may have caused and appreciate the reviewer for bringing this to our attention.

- *Figures 6h and k: Please explain why do NCT Anti-macrophages show higher glucose and lactate uptake than the Anti-macrophages from the control group, while the size of tumors is the lowest in NCT Anti-macrophages in vivo?*

Thank you for your insightful comment. The observation that NCT Anti-macrophages exhibit higher glucose and lactate uptake while the tumor size is lowest could be attributed to the metabolic reprogramming induced by chemotherapy. It is possible that the enhanced metabolic activity in Anti-macrophages, characterized by increased glucose and lactate uptake, is linked to a more aggressive anti-tumor response in the NCT group. This heightened metabolic activity could reflect an increased energy demand necessary for sustaining enhanced immune functions, ultimately contributing to the reduction in tumor size. We will expand upon this explanation in the revised manuscript to provide a clearer interpretation of these findings.

- *The supplementary Table 1 needs a better legend/more explanation.*

Thank you for your valuable feedback. We have revised the legend for Supplementary Table 1 to provide a more detailed explanation of its contents.

- *No tSNE plot showing epithelial cells colored by patient, which may be important for observation of cell heterogeneity, especially in the epithelial cell population.*

Thank you for pointing this out. We agree that a tSNE plot showing epithelial cells colored by patient would be valuable for observing cell heterogeneity within the epithelial population.

- *Several acronyms not explained in the text (for example GSVA, NMF).*

Thank you for bringing this to our attention. We have ensured that all acronyms, including GSVA (Gene Set Variation Analysis) and NMF (Non-negative Matrix Factorization), are clearly defined in the text at their first mention.

- *Availability of data and material section: Please describe "other experimental data" in more detail.*

Thank you for your suggestion. We have expanded the "Availability of Data and Material" section to provide a more detailed description of the "other experimental data" referenced. This will include specific types of data generated, their formats, and how they can be accessed by other researchers. This clarification will enhance transparency and facilitate the reuse of our data by the research community.

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