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Research Article

Hapten-Mediated Chemical Immune Therapy with Drug Administration into Cervical Cancer without Hysterectomy:Awakening Immune Cells for Immunotherapy

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Abstract

Background: Cervical cancer is one of the most common and deadly cancers in women, for whom with persistent infection of High-Risk Human Papillomavirus (HPV). Current treatment of cervical cancer involves radical hysterectomy, radiotherapy and chemotherapy or a combination.

Objective: We to define if Hapten Enhanced Intratumoral Chemotherapy (HEIC) was effective in boosting immunity for effective treatment of cervical cancer lesions.

Study Design: We used Single-Cell RNA Sequencing (scRNA-Seq) to obtain transcriptome profiles of 40239 cells from biopsies of cervical cancer lesions at the cervix directly from one patient before the start of HEIC and about 1 week after HEIC. The blood samples were taken at same time as biopsy. We compared the expression characteristics of malignant epithelial cells and immune cells, including Epithelial Cells, Ecs, Fibroblasts, Mural Cells, Tcells, Bcells, TandNK Neutrophils, Mast Cells, MPs, and Platelets, as well as the dynamic changes in cell percentage and cell subtype heterogeneity.

Results: Intratumoral injection of chemotherapy drug plus hapten induces acute immune response in cervical cancer lesions, and further awakens immune cells.

Conclusion: HEIC provides a potential treatment method for cervical cancer tailored to each patient's condition.

Introduction

Cervical cancer is one of the most common cancers threatening women's health, that is closely linked to the persistent infection of high-risk Human Papillomavirus (HPV) [1,2]. Approximately 90% of cervical cancer occurs in low-income and middle-income countries due to the lack of organized screening and HPV vaccination programs in these countries. In high-income countries, implementation of screening and vaccination has reduced the incidence rate and mortality of cervical cancer by more than half in the past 30 years. Treatment of cervical cancer depends on the severity of the disease at the time of diagnosis and the availability of local resources, which may include radical hysterectomy, radiotherapy and chemotherapy, or a combination of both, which has become a standard of treatment [2]. Advanced inoperable cervical cancer is a challenging entity due to the increased percentage of local and distant recurrences. In addition, recurrent cervical cancer and new metastatic disease that cannot be cured are considered incurable and have a poor prognosis. Well-designed clinical trials conducted over the past 30 years have revealed effective chemotherapy drugs and their optimal combinations [3].

The current standard treatment for cervical cancer includes radiation, chemotherapy, and/or surgical removal, due to the lack of intervention in the form of immunotherapy, local recurrence after treatment is still an unresolved issue. However, standard treatments have significant side effects and are of limited efficacy against advanced disease and relapsed or metastatic cases. Immunotherapy offers new hope, as evident by the recent approval of programmed cell death protein-1 blocking antibodies for relapsed or metastatic disease [4]. Some of the novel modalities are also being evaluated in combination with standard platinum-based chemotherapy regimen. At this time, pembrolizumab is approved for the treatment of relapsed or metastatic Programmed Death Ligand 1 (PD-L1) positive cervical cancer after frontline chemotherapy treatment. Multiple novel therapeutic modalities are emerging as safe and effective for the treatment of cervical cancer patients. [5]. There are several types of immune-related drugs that have been attempted to activate the immune system to improve treatment outcome. These include immune checkpoint inhibitors, therapeutic vaccines, engineered T cells, and antibody drug conjugates. Checkpoint inhibitors appear to be the best treatment methods for research, with encouraging Phase II studies in established environments. Vaccines and engineered T cells that use unique immune activation mechanisms are usually in the early stages of development [6,7].In the current study, we aimed to determine if Hapten Enhanced Intratumoral Chemotherapy (HEIC) was effective in treating cervical cancer. We hypothesized that HEIC can induce acute immune response to control both cervical cancer and prevent recurrence of cervical cancer, HEIC is used for treating several cancers by hapten modified tumor associate antigens [8-10].

Biopsy of cervical cancer lesions was carried out using forceps at 12, 3, 6, and 9 o'clock at the cervix directly from one patient before the start of HEIC and about 1 week later after HEIC. We then used Single-Cell RNA Sequencing (scRNA-Seq) to obtain transcriptome profiles of 45373 cells. Through comparative analysis of different samples of CIN, we comprehensively described the expression characteristics of 10 cell types including Epithelial cells(EpithelialCells), epithelial cells(epithelialcells), stromal cells(stromal cells) : Endothelial cells(ECs), Fibroblasts, Mural cells(MuralCells), immune cells: B cells(BCells), Plasma cells(PlasmaCells), T and NK cells(TandNK), Mast cells(MastCells), Mononuclear phagocytes(MPs), Erythrocytes., as well as the dynamic changes in cell percentage and cell subtype heterogeneity. Our results provide evidence that intratumoral co-administration of HEIC induced acute immune response in cervical cancer lesions to prevent their abnormal proliferation.

Materials and Methods

Clinical Specimens

The three patients had a pathological diagnosis and were determined to have a clinical stage of cervical cancer lesions [7,8]. The patient did not have any other therapy before this study. Before receiving treatment at Beijing Baofa Cancer Hospital, the patient's physical condition was evaluated and determined to meet the indications for HEIC. This experimental treatment was approved by the hospital ethics committee (TMBF 0010, 2015) in accordance with relevant guidelines and regulations. After the patients prepared for biopsies collected, cleaning and disinfection of perineum and vagina under general anesthesia and a disinfecting towel is laid. When cervical cancer lesions was seen, the four of small piece of cervical cancer lesions (1.5mm×1.5mm×2mm) was taken at 12, 3, 6, and 9 o'clock at the cervix as an untreated sample for scRNA-Seq analysis; This was followed by intratumoral injection at 12, 3, 6, and 9 o'clock at the cervix of a total of 10 ml that contained 1.00 mg/ml Adriamycin (Adr), 0.80 mg/ml of cytarabine (Ara-C), 20.0 mg/ml of H_2O_2 and 144 mg/ml of penicillin as the hapten [9,10,11]. 1 week post injection, biopsies of cervical cancer lesions carried out again using forceps at 12, 3, 6, and 9 o'clock at the cervix, another four small pieces of precancerous cervical lesions (1.5mm×1.5mm×2mm) as treated samples for scRNA-Seq analysis.

Tissue Disassociation and Cells Collection

After small cervical lesions tissues and blood samples were collected, the fresh tissue samples were immediately stored in the

sCelLiVE® Tissue Preservation Solution (Singleron) on ice. The tissues were cut into small tissue pieces and were transferred to a 15-ml centrifuge tube, followed by digestion using sCelLiVE® Tissue. Dissociation Solution (Singleron) at 37°C for 15 min with shaking. The samples were then filtered with 40 µm sterile strainers, and centrifuged at 1,000 rpm at 4°C for 5 min. Next, 2 ml GEXSCOPE® red blood cell lysis buffer (RCLB, Singleron) was added to lyse the red blood cells for 10 min. Finally, the single cell suspension was collected after re-suspension with PBS, and trypan blue (Sigma) staining was used to calculate cell activity and cell count under a microscope.

Single-Cell RNA Sequencing

Single-cell suspensions ($1\sim3\times10^5$ cells/mL) in PBS (HyClone) were loaded onto microwell chip using the Singleron Matrix® Single Cell Processing System. Briefly, the scRNA-seq library was constructed using the GEXSCOPE® Single Cell RNA Library Kits (Singleron). The library was lastly sequenced with 150 bp was diluted to 4nM and paired-end reads on the IlluminaHiSeq X platform following an established protocol [25]. Sequencing data processing and quality control was performed as described in previous publications [12].

Differentially Expressed Genes (DEGs) Analysis

To identify differentially expressed genes (DEGs), genes expressed in more than 10% of the cells were selected in both groups of cells and with an average log (fold changes) value greater than 1 as DEGs. Adjusted p value was calculated by the Benjamini-Hochberg correction. The P value of 0.05 was used as the criterion to assess the statistical significance.

Cell Type Annotation

The cell type identity of each cluster was determined with the expression of canonical markers found in the DEGs using SynEcoSys database (Singleron Biotechnologies). Heatmaps/dot plots/violin plots displaying the expression of markers used to identify each cell type were generated by the Scanpy built-in functions and ggplot2.

Single-Cell Copy Number Variation (CNV) Analysis

The InferCNV package was used to detect the CNAs in malignant cells. Non-malignant cells (T and NK cells) were used as control references to estimate the CNVs of malignant cells. Genes expressed in more than 20 cells were sorted based on their loci on each chromosome. The relative expression values were centered to 1, using 1.5 standard deviation from the residual-normalized expression values as the ceiling. A slide window size of 101 genes was used to smoothen the relative expression on each chromosome, to remove the effect of gene-specific expression.

Pathway Enrichment Analysis

To investigate the potential functions of DEGs between clusters, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used with the "clusterProfiler" R package 3.16.1. [13]. The GO gene sets including Molecular Function (MF), Biological Process (BP), and Cellular Component (CC) categories were used as references. Pathways with the adjusted p value less than 0.05 were considered as significantly enriched.

Trajectory Analysis

Monocle 2 algorithm [14] was used for pseudo-time trajectory analysis, and the dimensionality reduction method used was DDRTree.

Intra-Tumoral Heterogeneity (ITH) score calculation

The ITH score was defined as the average Euclidean distance between the individual cells and all other cells, in terms of the first 20 principal components derived from the normalized expression levels of highly variable genes. The highly variable gene was identified using the "FindVariableGenes" function in the Seurat package, with default parameters.

### Cell-Cell Interaction Analysis (CellPhoneDB)

Cell-Cell Interaction (CCI) between B cells, Epithelial cells, Fibroblasts, Mononuclear phagocytes, Mast cells, Neutrophils, T and NK cells were predicted based on known ligand–receptor pairs by Cellphone DB v2.1.0 [15,16,17].

Results

Clinical Benefit Characteristics

In biopsies taken post-treatment, pathological examination confirmed early diagnosis as cervical cancer. Follow-up examination every four weeks after the treatment during a six-month period, physical examination and CT of the patient showed no signs of cervical cancer lesions in smooth surface of cervix and the patients was in good health living a normal life average over 1.2 years, no recurrence and metastasis. Landscape of single cell transcriptome sequencing before and after precancerous lesions treatment Single cell transcriptome sequencing was performed on two cervical cancer tissues before and after treatment. After dimension reduction and clustering, The cell atlas of 3 samples was constructed, with a total of 45,373 cells and 10 cell types, including Epithelial cells (EpithelialCells), Epithelial Cells (epithelialcells) and Stromal Cells(stromal cells) : Endothelial Cells(ECs), Fibroblasts, Mural Cells (MuralCells), Immune Cells: B Cells (BCells), Plasma Cells (PlasmaCells), T and NK Cells (TandNK), Mast Cells(MastCells), Mononuclear Phagocytes(MPs), Erythrocytes. Epithelial, T/NK

and Mononuclear Phagocytic (MP) cells were the main cell components, and stromal cells and B lymphocytes were gradually reduced after treatment (Figure 1).
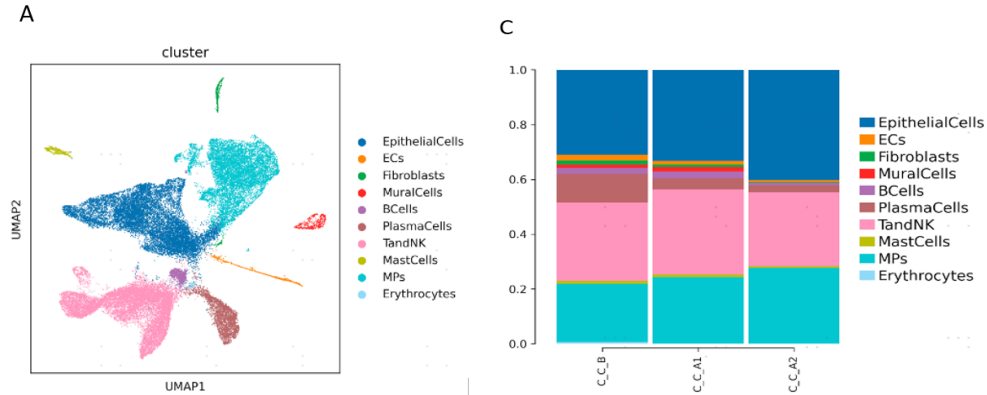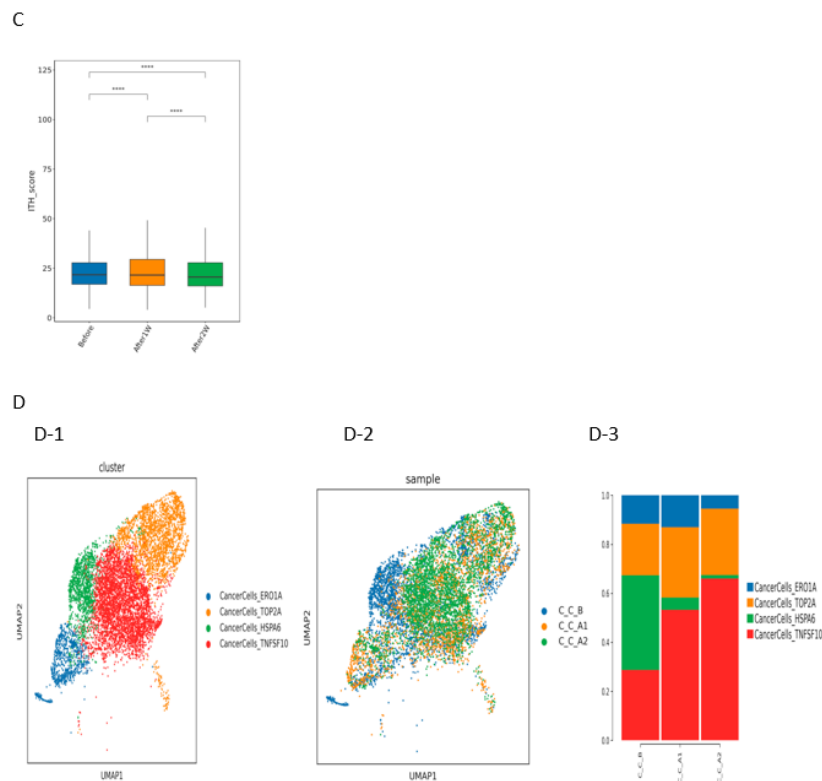

Figure 1 : A: Color clustering UMAP by cell type, Color clustering UMAP by sample; **B:** Single sample cell composition histogram.

Changes in Epithelial Cells Before and After Treatment of Cervical Precancerous Lesions

EpithelialCells- Tumor cell identification: inferCNV results showed that there was significant amplification of chromosomes 1,3,18 and significant deletion of chromosomes 13,14,17 in epithelial cells. scCancer results showed that epithelial cells had a high malignant score, and all red epithelial cells were evaluated as tumor cells (Figure 2 A,B). The ITH heterogeneity of CancerCells decreased significantly after 2 weeks of treatment: Cancer cells accumulate somatic mutations during division and proliferation, some of which bring more adaptive advantages to cancer cells, and may form genetically different tumor cell populations, and specific tumor cell subpopulations carry specific subclonal mutations, which is called Intra-Tumor Heterogeneity (ITH) (Figure 2C). After treatment, the tumor heterogeneity score decreased, indicating that the malignant degree of the tumor decreased, which was consistent with the clinical effect. The Hallmark scores for CancaerCells before and after treatment is presented in (Figure 2D) The data show that after treatment, tumor cell hypoxia, estrogen response, angiogenesis, MYC target v2, mTORC1 signal, EMT transformation, unfolded protein response and other scores were decreased, which may be related to the effective treatment.
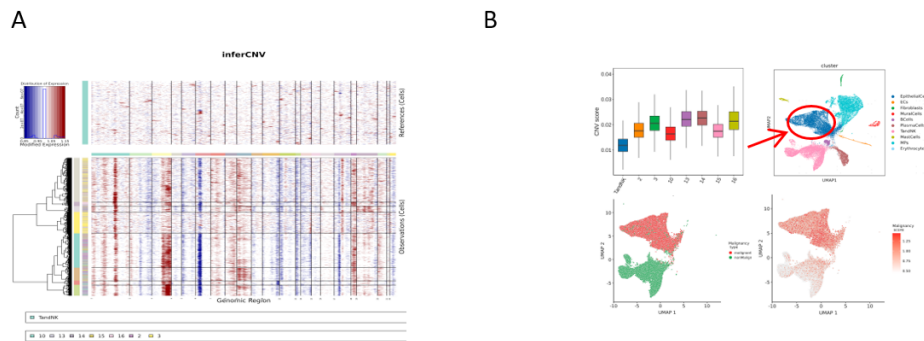
Figure 2: A. Epithelial cell inferCNV results; B. Epithelial cells inferCNV and scCancer results; C. Cancer Cells ITH heterogeneity; D. D1: Color clustering UMAP by cell type; D2: Color clustering UMAP by sample; D3: Single sample cell composition histogram.
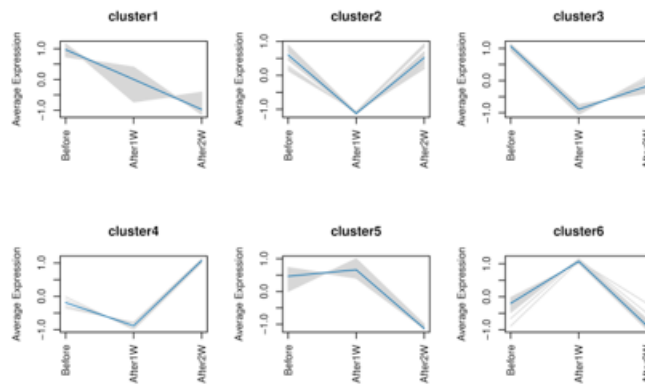
CancaerCells HallMark Gene set score details: Because the rapid and uncontrolled proliferation of tumors limits the availability of oxygen, inadequate blood supply or hypoxia is a typical microenvironmental feature of almost all solid tumors (Figure 2E). Tumor cells adapt to hypoxia while resulting in a more aggressive and treatment-resistant tumor phenotype. (10.1186/ S12943-019-1099-9). The occurrence of cervical cancer is mainly due to the infection of Human Papilloma Virus (HPV), estrogen and estrogen may be the development of cervical cancer risk factors (10.1016 / j.b iopha. 2016.11.007). Angiogenesis in tumor metastasis and progress plays a vital role, this is because the tumor cell growth and survival also need of vascular system provides oxygen and nutrients (10.1016 / j. jphar. 2021.174021) MYC is a transcription factor known to regulate multiple genes that promote cell growth and proliferation (Figure 2F), and a high MYC target v2 score in primary breast cancer is associated with poorer survival. (10.3390/ijms21218127). Existing studies have shown that mTOR a gene activation has important influence on the occurrence of cervical cancer development (10.1016 / j.y gyno. 2009.12.020), breast cancer in mice model, Inhibiting mTORC1 signaling in cancer cells reduced tumor growth (10.1038/s41586-023-06256-5). Tumor cells invade and metastasise through Epithelial-To-Mesenchymal Cell Transformation (EMT) (10.1155/2021/9918379). The Unfolded Protein Response (UPR) of eukaryotic cells is a network of signal transduction pathways to relieve the load of unfolded or misfolded proteins and restore protein homeostasis. UPR activation promotes the oncogenic transformation process, in which all UPR signaling branches contribute to tumor growth, angiogenesis, and immune evasion (10.1038/s41580-020-0250-z). The tumor MHC score was significantly increased after treatment: Classical MHC I molecules provide antigen-peptide ligands to CD8 T cells, and expression of MHC I in tumors promotes elimination of CD8+ T cell-dependent cancer cells (Fig. 2 G) (10.1038/ cmi.2014.105, 10.1158/2159-8290.CD-20-0812). Increased expression of classical MHCI in tumor cells after two weeks of treatment may help CD8T cells to recognize and kill tumor cells, which is related to the effectiveness of treatment. MHC-II is critical for antigen presentation to CD4+T lymphocytes (10.1158/1078-0432.CCR-18-3200), and in triple-negative breast cancer, Tumor cell expression of MHCII is associated with good prognosis and lymphocyte infiltration (10.1158/2326-6066.CR-15-0243). The MHCII score of tumor cells increased after treatment, which may be related to the effective treatment.
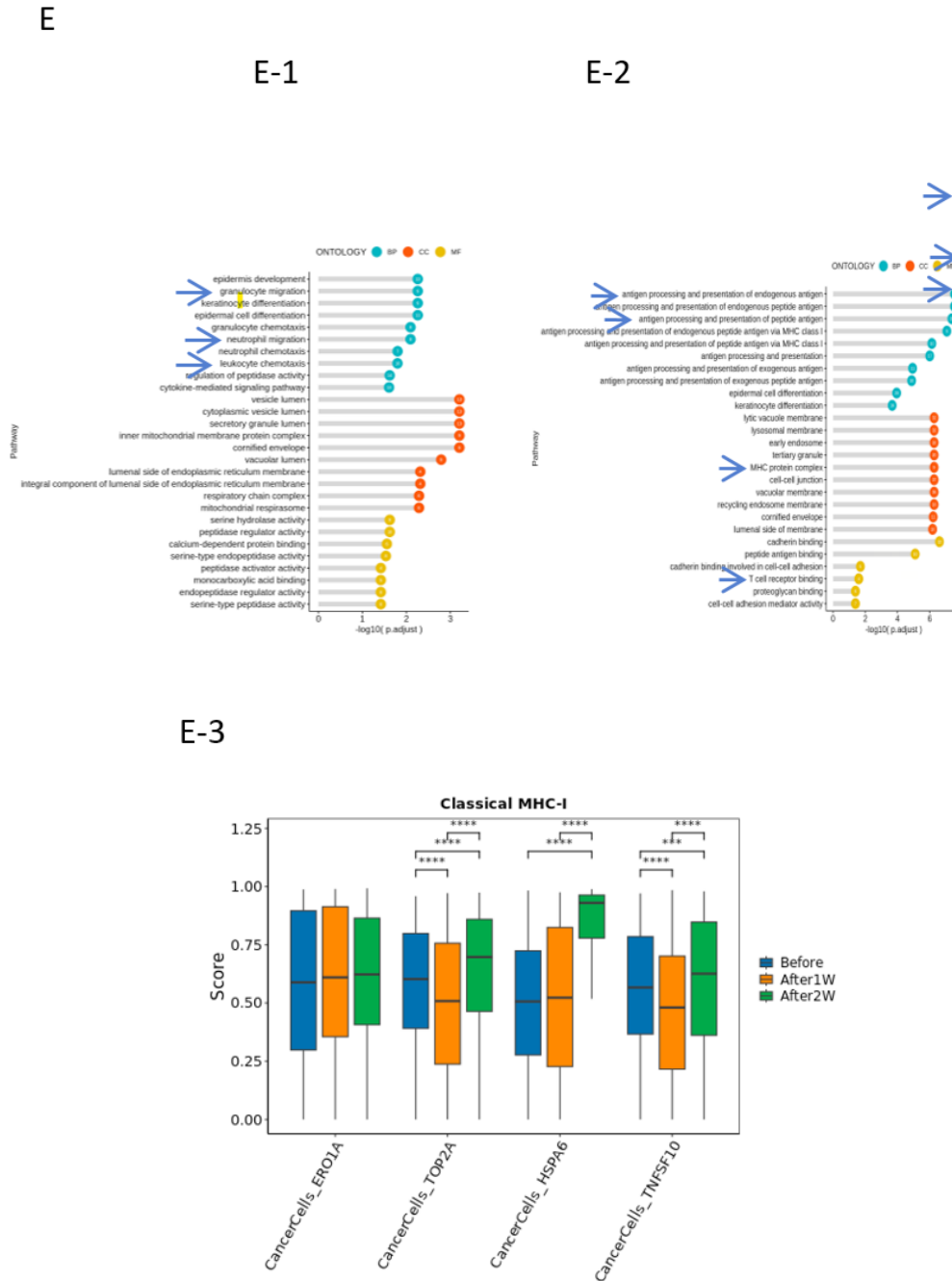
Figure 2: E E1：After1W vs Before CancerCells_HSPA6 GO enrichment；E2：After 2W vs Before CancerCells_HSPA6 GO enrichment；E3：CancerCells subtype MHC-associated Ucell score F. CancaerCells HallMark gene set score

EpithelialCells cell subdivision - dimension reduction (Figure 2 H:a,b,c): A total of 8773 EpithelialCells were epithelialcells, and four different subtypes were obtained by subdivision. CancerCells_ERO1A subtype raised ERO1A and tumor specific antigen CEACAM5/6, ERO1A express more during endoplasmic reticulum stress, help tumor cells survive (10.1016 / j.x CRM. 2023.101206). CancerCells_ HSPA6 subgroup highly expressed tumor suppressor genes SERPINB5, CDKN1A and CDKN2B. It was suggested that the up-regulation of CDKN2B could prevent the formation of teratoma in polyfibromodulin reprogramming cells (10.1172/JCI125015). The proportion of CancerCells_HSPA6 subgroup decreased significantly after treatment, which may be resulted from the effective treatment. CancerCells_ TOP2A overexpressed the cell cycle related genes TOP2A and MKI67, and was in a state of proliferation.
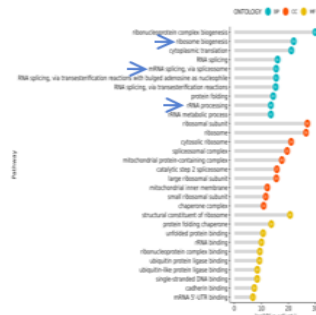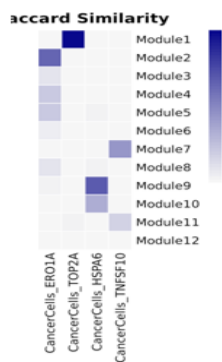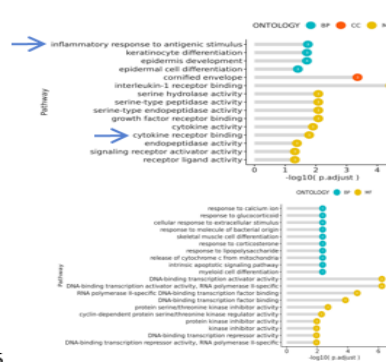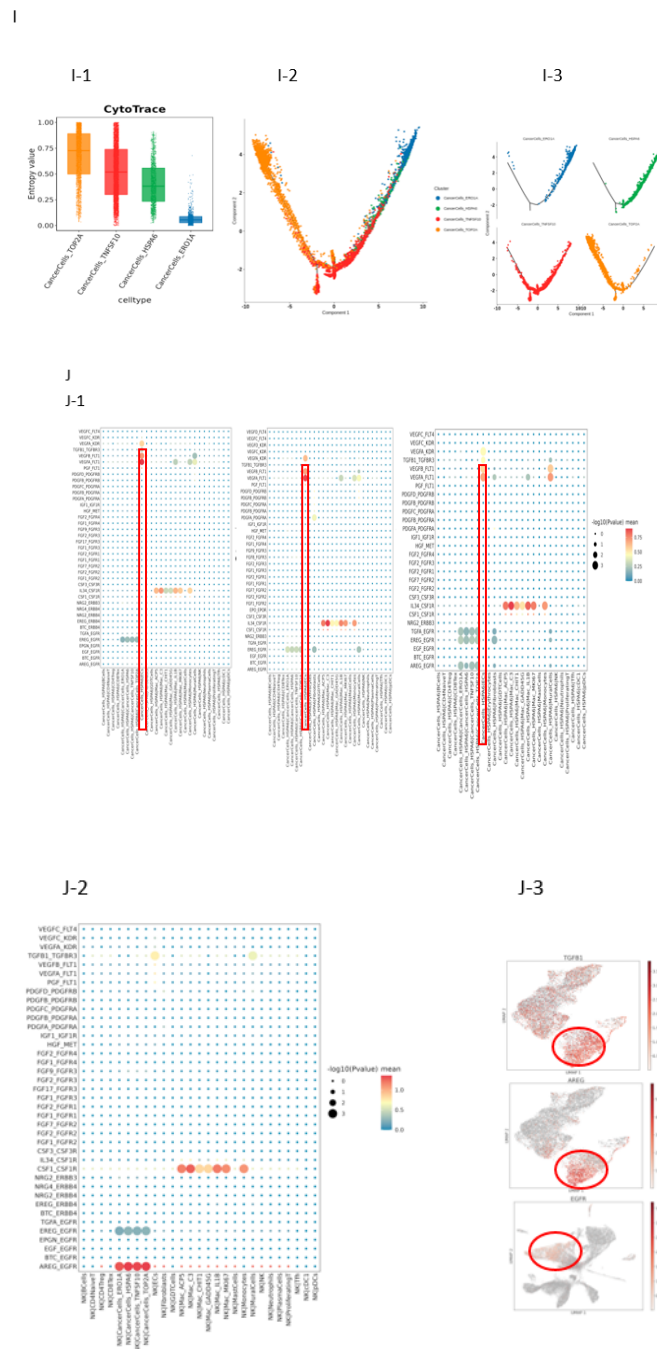
Figure 2: G. CancaerCells HallMark gene set score. H.HallMark score of CancerCells before and after treatment (hypoxia, estrogen response, angiogenesis). H1:Gene expression pattern analysis of CancerCells_HSPA6 subpopulation； H2;Gene expression pattern analysis of CancerCells_HSPA6 subpopulation. I I1;CytoTRACE analysis of tumor cell subtypes;I2;monocle analysis of tumor cell subtypes; I3;monocle analysis of tumor cell subtypes. J1;Cell interactions were shown in groups (Before,After1W,After2W);J2;Cell interactions were shown in groups (Before,After1W,After2W);J3;TCFB1 and AREG are highly expressed in NK cells,EGFR is highly expressed in tumor cells

**Tandnk Changes in Cervical Cancer Lesions Before and After Treatment**

TandNK cell subdivision - Dimension reduction /Top10 gene: There were 11553 T and NK cells in total, and 7 different subtypes were obtained by subdivision annotation. Including Proliferating T Cells(ProliferatingT), Gamma Delta T Cells(GDTCells), Natural Killer Cells(NK), Follicular Helper T Cells(Tfh), CD4+ Naive T Cells(CD4NaiveT), CD4+ Regulatory T Cells(CD4Treg), CD8+ Exhausted T Cells(CD8Tex)Figure 3A). After treatment, the proportion of toxic functional subtypes such as GDTCells and NK increased, while the proportion of CD4NaiveT and immunosuppressive CD4Treg decreased. The toxicity and depletion of some TandNK subtypes were enhanced after treatment: ProliferatingT, GDT, NK, CD8Tex subtype toxicity score increases after treatment, and NK and CD8Tex depletion ability increases after treatment. Enhanced T-cell toxicity can help to inhibit tumor progression and even eventually eliminate tumors (Figure 3B). Liu et al also observed an increased NK cytotoxicity score after cervical cancer chemotherapy, and the expression of cytotoxic genes may be associated with a better prognosis for patients (10.1038/s41392-022-01264-9). Tumor specific CD8 + T cells in the "failure" of the dysfunctional state associated with persistent antigen stimulation (10.3389 / fimmu. 2020.622509). Proliferating cells are mainly CD8TCells and NK (right).
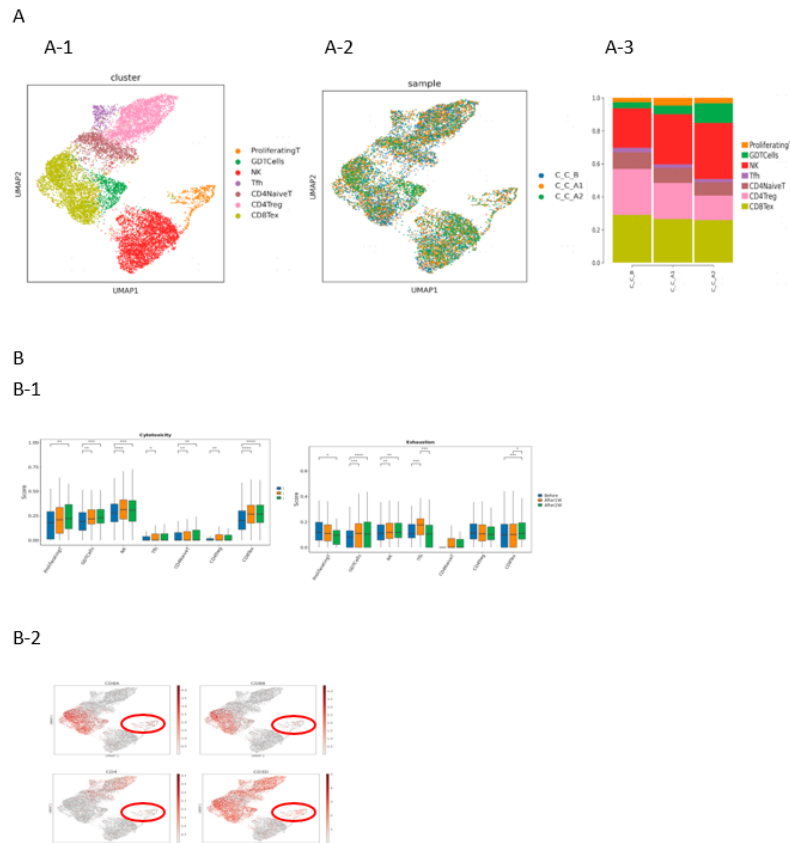

Figure 3: A. A1:Color clustering UMAP by cell type; A2:Color clustering UMAP by sample; A3; Sample cell composition histogram. B. B1; Toxicity and depletion capacity Ucell scores of TandNK subtype; B2; ProliferatingT gene expression feature plot

After treatment, ProliferatingT is enriched to antigen processing and peptide antigen presentation, leukocyte mediated immune positive regulation, lymphocyte mediated immune positive regulation, and cytokine production involved in immune response positive regulation. After treatment, NK subpopulation was significantly enriched into the pathways of positive regulation of lymphocyte activation, positive regulation of chemokine production, leukocyte cell adhesion, tumor necrosis factor binding, and T cell receptor binding. After treatment, GDT cells were significantly enriched in viral response, positive regulation of immune processes and other pathways. These results suggest that the positive immunoregulatory ability of T/NK cells is enhanced after treatment (Figure 3 C).

C
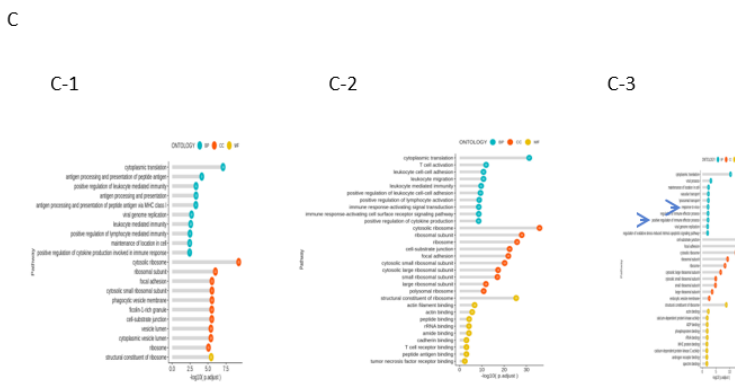
Figure 3: C. C1; After 2W vs Before Proliferating T GO enrichment; C2; After 2W vs Before NK GO enrichment; C3; After 2W vs Before GDT GO enrichment.

After two weeks of treatment, the expression of NK cells XCL1 and CCL5 was enhanced, effectively inducing dendritic cells and CD8+ T cell responses (Figure 3 D) XCL1 is mainly produced by activated NK and infiltrates tumors with chemotactic dendritic cells (10.3389/fimmu.2018.02775). CCL5 chemotactic T cells enter the tumor and cause an immune response. Chemokine CCL5 and CXCL9 associated with the CD8 + T cell infiltration of solid tumor (10.1016 / j.carol carroll cell. 2019.05.004).
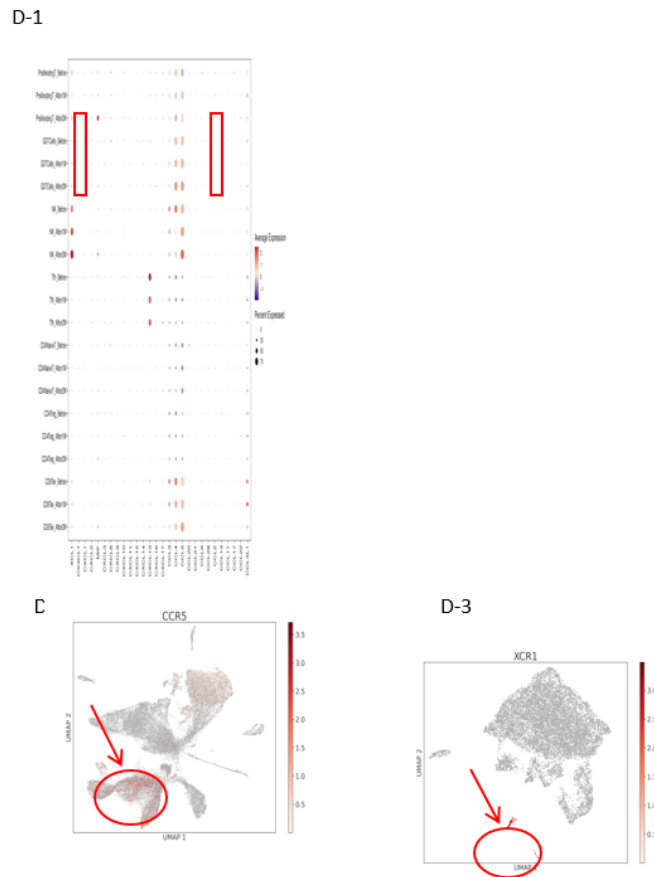
Figure 3: D.D1; TandNK subtype chemokine expression site map; D2; Expression of CCL5 receptor CCR5 (mainly expressed by T cells); D3; Expression of XCL1 receptor XCR1 (mainly expressed by dendritic cells)

Changes of Mps Cells in Cervical Cancer Before and After Treatment

MPs totaled 8633 cells, and 6 different subtypes were obtained by subdivision annotation. Including Proliferating macrophages(Prolife ratingMacro), Neutrophils, Macrophages, Monocytes, Conventional type 1 dendritic cells(cDC1), Plasmacytoid dendritic cells(pDCs) (Fog. 4 A) Macrophages accounted for more cells, and there was no significant difference in cell types between groups. Macrophages consist of 7780 cells divided into 6 heterogeneous subpopulations (Fig. 4 B B-a). The Mac_IL1B and Mac_GADD45G subgroups favored the pro-inflammatory M1 phenotype, while the remaining subtypes favored the anti-inflammatory M2 phenotype (Figure 4 B B-b). Mac_CHIT1 subgroup highly expressed macrophage activation marker CHIT1 (10.1158/1078-0432.CCR-19-3372), which was enriched to the antigen presentation related pathway. The Mac_IL1B subgroup up-regulates several chemokines CXCL2/3/8 and CCL20, which play a pro-inflammatory role (Figure 4C). The Mac_C3 subgroup highly expressed the complement component C3 and the pro-inflammatory gene CXCL9/10, suggesting an enhanced ability to mediate complement immunity and an enhanced ability to infiltrate chemotactic T cells and monocytes, promoting anti-tumor immunity (10.1038/s41392-022-01264-9). Mac_ACP5 subgroup ACP5 high expression of lipid related genes, APOC1 and chemokines CCL18, lipid metabolic control the phagocytosis of macrophage, and the production of inflammatory cytokines, and has antiviral function (10.1016 / j.t. cb. 2020.09.006). Mac_GADD45G subgroup up-regulates stress response genes GADD45G,HSPA1A, enrichment of unfolded protein response, heat shock protein binding and other pathways. The Mac_MKI67 subgroup upregulates the cell cycle associated protein MKI67,TOP2A, in a proliferative state.

After treatment, the pro-angiogenesis score of each subgroup was decreased, and the therapeutic and pro-angiogenesis ability of Mac_C3 subgroup and Mac_GADD45G subgroup was significantly decreased, which inhibited tumor growth and metastasis (Figure 4D). The ability of medullary cells to promote angiogenesis is essential for wound healing under normal physiological conditions, but the function of medullary cells to promote angiogenesis contributes to tumor progression under pathological conditions (10.3390/ijms19092565). To promote angiogenesis is associated with a variety of poor clinical outcome of cancer (10.1016 / j.carol carroll ell. 2021.01.010). Compared with before treatment, after 2 weeks of treatment, Mac_C3 subgroup mainly enriched into antigen processing and presentation, MHC protein complex, antigen peptide binding and other pathways.

Compared with before treatment, after 2 weeks of treatment, the Mac_IL1B subgroup was significantly enriched into antigen processing and presentation, positive regulation of leukocyte adhesion, MHC protein complex, MHC class II protein complex, antigen peptide binding and other pathways. Compared with before treatment, after 2 weeks of treatment, Mac_ACP5 subgroup mainly enriched MHC Class II presentation, MHC protein complex binding, antigen processing and presentation, MHC II receptor activation and other pathways. These results suggest that the antigen presenting ability of macrophages is enhanced after treatment (Figure 4E).

After treatment, monocyte expression of CXCL9 was enhanced, which was conducive to T cell recruitment (Figure 4 F). A study in a mouse model of breast cancer found that chemotactic NK cells and CXCR3+T cells with high expression of Cxcl9 inhibited local tumor growth and lung metastasis (10.1002/cam4.934). After 2 weeks of treatment, CXCL16 expression was enhanced in macrophages, CXCL16 chemotactic T cells, and NKT cells (10.3390/ijms22073490).

Changes in Fibroblast Group Before and After Treatment for Cervical Precancerous Lesions

Subdividing the subpopulations of Fibroblasts cells gave rise to a total of 4 cell types (Figure 4a), including Fibroblasts_LUM; Fibroblasts_ POSTN; Fibroblasts_ ACTA2 and Fibroblasts_ IGFBP2 cell subpopulation. Analysis the proportion of each cell found that after drug treatment, fibroblasts in the tissue. The proportion of IGFBP2 cells significantly increased. The proportion of cells in the ACTA2 subgroup significantly decreased (Figure 4b). Heterogeneity exists between various subpopulations of fibroblasts (Figure 4c). Further analysis of Fibroblasts_ Differential gene analysis of IGFBP2 cells between groups: Fibroblasts after treatment_ IGFBP2 cells overexpress multiple chemokines and interleukins IL24, IL19, and CCL8 (Figure 4 d,e), and upregulate the receptor signaling pathway of JAK-STAT γ Interferon response, NIK/NF- κ Inflammatory related pathways such as B signaling pathway and type I interferon signaling pathway (Figure 4f). Time series analysis showed that there were differences in the differentiation of fibroblasts in tissues before and after treatment. Before treatment, the cells in the sample were overall located in the early and middle stages of the trajectory, while after treatment, the cells were overall located in the middle and late stages (Figure 4i); Before treatment, Fibroblasts_ACTA2 is in the early stage of differentiation, Fibroblasts_IGFBP2 is in the middle and late stages of differentiation, and after treatment, Fibroblasts_ ACTA2 cell reduction, Fibroblasts_ IGFBP2 increases (Figure 4g,h), indicating that Fibroblasts after treatment_ ACTA2 may differentiate into Fibroblasts_ IGFBP2 subgroup. Further score the characteristic gene sets of adipose derived fibroblasts, inflammatory fibroblasts, myofibroblasts, and epidermal promoting fibroblasts for each subgroup of fibroblasts (Figure 4g).
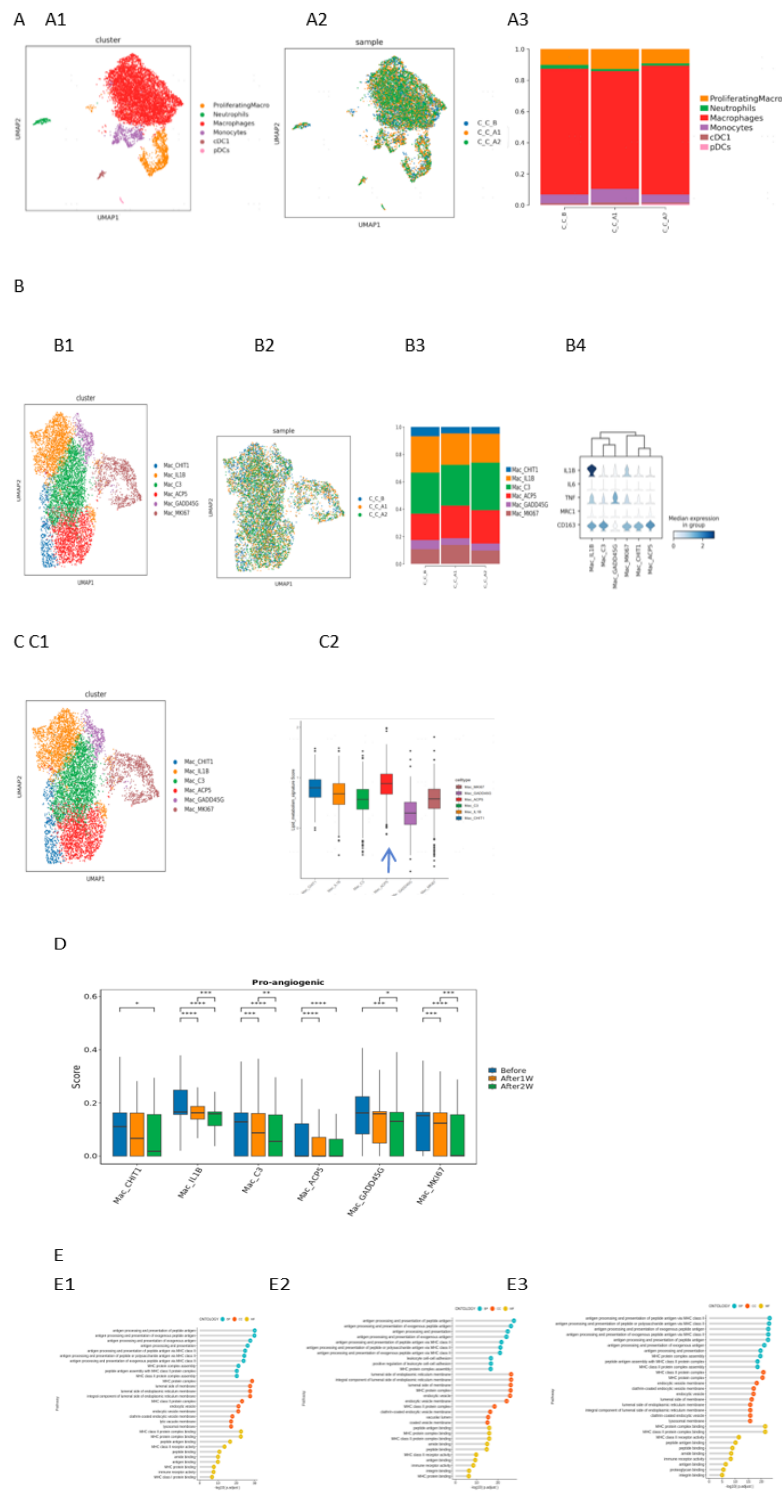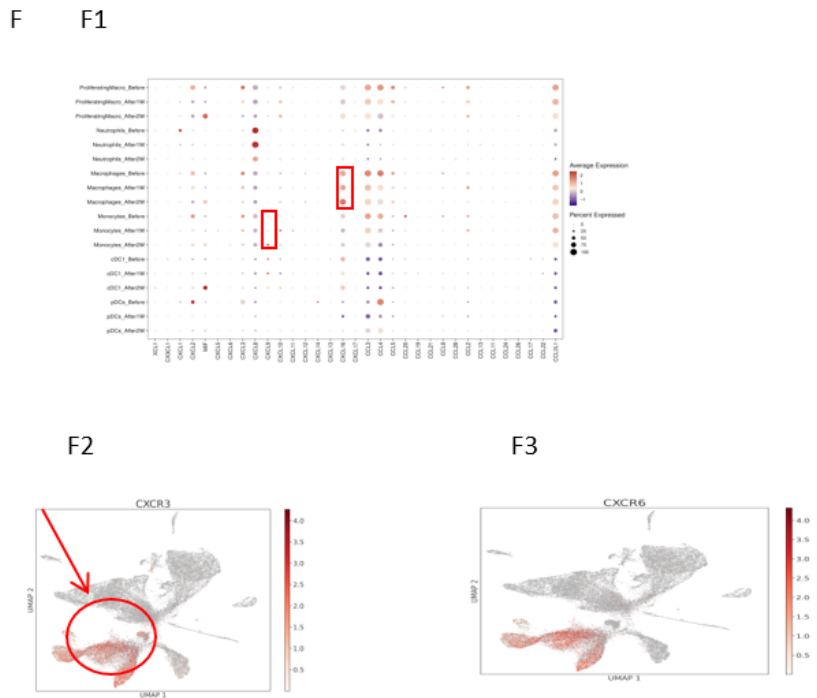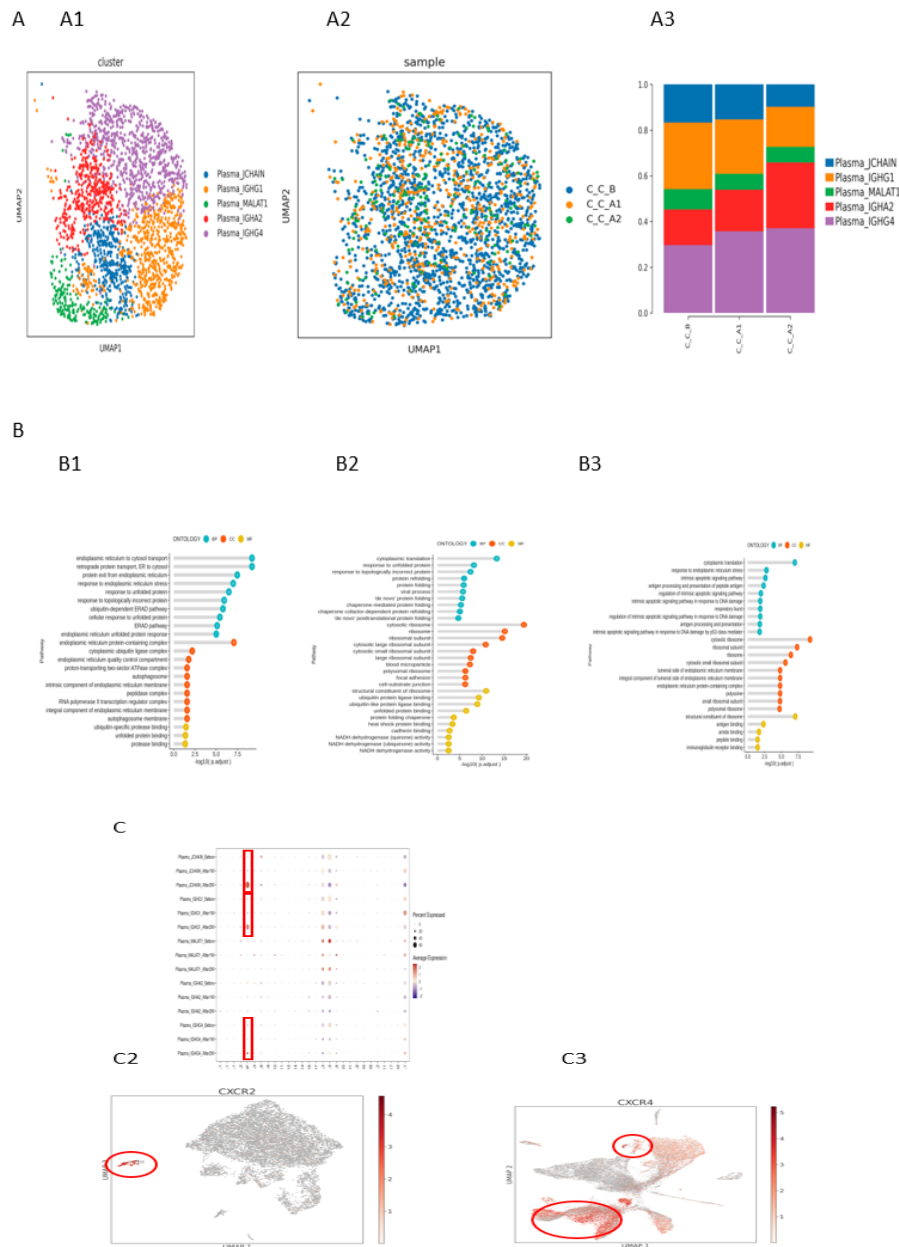
Figure 4: A A1:Color clustering UMAP by cell type; A2:Color clustering UMAP by sample; A3:Single sample cell composition histogram. B B1:Color clustering UMAP by cell type; B2:Color clustering UMAP by sample;B3:Single sample cell composition histogram; B4:M1 and M2 characteristic gene map; C C1:Color clustering UMAP by cell type; C2:Lipid metabolizing macrophage characteristic gene set score, D:Ucell score of macrophage subtype promoting angiogenesis. E E-1:After 2W vs Berfore Mac_C3 GO enrichment map; E2:After 2W vs Berfore Mac_IL1B GO enrichment map; E3:After2W vs Berfore Mac_ACP5 GO enrichment map. F F1:MPs subtype chemokine expression site map; F2:Expression of CXCL9 receptor CXCR3 (expressed by T cells); F3:Expression of CXCL16 receptor CXCR6 (expressed by T cells).

Changes in Cellular Communication Before and After Treatment of Cervical Precancerous Lesions

Compared to cell interaction analysis showed that the signal communication between cells decreased overall in the tissues before and after treatment (Figure 5a). Compared to that in PBMC samples before and after treatment: ClassicalMono_1. enhanced communication with other immune cells (Figure 5b). Cell interaction analysis in the tissues showed that before and after treatment: ClassicalMono_2/ ClassicalMono_3; ClassicalMono_2/MatureDC; ClassicalMono_2/cDC2, CCL3 between the above cells_CCR1; CCL3. The signal communication between the CCR5 receptor gene pairs is weakened (Figure 5c), which is similar to the previous results . On the contrary, Fibroblasts_ IGFBP2|Fibroblasts_ACTA2; IL24_NOTCH2 signal enhancement (Figure 5c,d), Fibroblasts (Figure 5e)._ Upregulation of IL-24 expression was seen in IGFBP2 cells. Compared to that in PBMC samples before and after treatment: ClassicalMono_1|cDC2; ClassicalMono_2|cDC2; LGALS9_Enhanced signal communication between HAVCR2 (Figure 5d).

Figure 5: A. A1:Color clustering UMAP by cell type; A2:Color clustering UMAP by sample; A3:Single sample cell composition histogram. B. B1:After 1W vs Berfore Plasma_IGHA2 GO enrichment diagram; B2; After 2W vs Berfore Plasma_IGHA2 GO enrichment diagram; B3; After 1W vs Berfore Plasma_IGHA2 GO enrichment diagram. C. C1:Comparison between PlasmaCells subtype chemokines; C2; MIF chemotactic neutrophils by CXCR2; C3; MIF is chemoattractant to T monocytes by CXCR4

Discussion

In this study, we used scRNA-Seq and demonstrated that precancerous cervical lesions (cervical intraepithelial neoplasia; CIN) can be treated HEIC therapy and provides evidence supporting that hapten enhanced intratumoral chemotherapy (HEIC) precancerous cervical lesions with HPV infection induced acute immune response to control the CIN and turning the HPV to negative. Our results showed the cells such as Bcells and TandNK increased in both PBMC and cervical tissues, while the proportion of stromal cells such as Ecs, Fibroblasts, and MuralCells decreased in tissues before and after treatment and the proportion of Neutrophils cells in the blood significantly decreased. The increase in immune cells may be due to the hapten with drugs kill precancerous cervical lesions with HPV and activating the immune cells, making it effective in activating the immune cells to attacking diseased cells. The decrease in stromal cells may be due to the drug affecting the tissue structure and function around the affected cells. These changes may indicate that drug and hapten therapy has had a positive impact on the therapeutic efficacy of CIN3, consistent in conjunction with clinical outcomes which is HPV turning to negative and cervical surface turning to smooth.

Significantly clinical benefit is that one local therapy with hapten and chemotherapy drugs can kill local precancerous cancer and hapten modified with tumor associated antigens and the major oncogenic protein expressed in HPV-associated precancerous cervical lesions, also induce immune response to fight both precancerous cancer cell and HPV virus. We used scRNAseq and demonstrated that HEIC treatment induces the interaction among Epithelial Cells, Ecs, Fibroblasts, Mural Cells, Tcells, Bcells, TandNK Neutrophils, Mast Cells, MPs, and Platelets promotes the expression of many genes contributing to the upregulation of immune response in precancerous cervical lesions. Since it is the first attempt to treat precancerous cancer positive for HPV, we will need a larger sample size to prove its effectiveness [18]. Detail

subdivision of Tfh cell subpopulations resulted in four cell types with Tfh1 cell in dominant group and enriches the phagocytic pathway while Tfh cell subpopulations increased, and function of follicular T cell subsets in heterogeneity. This is consistent with the proposed roles for phagocytosis in degradation of foreign pathogens or cell wastes, which play an important role in immune defense and metabolic regulation [19]. Our results also showed increased expression of CD52, EMP3, TMSB10, CCL5, TXNIP in the late stage of differentiation; CD52, mainly highly expressed in B cells and T cells, is an important immune regulatory factor for T cell activation [20]. Overexpression of CD52 leads to increased infiltration of M1 macrophages, monocytes, T-follicle helper cells, and resting memory CD4T cells. CCL5 gene encodes a chemokine ligand 5, which can promote the chemotaxis and aggregation of monocytes, eosinophils, basophils, T cells, natural killer cell and other immune cells, thus participating in the regulation of immune response and the mediation of inflammatory response [21]; TXNIP is a thioredoxin that can bind to Reactive Oxygen Species (ROS), avoiding ROS damage to cells and protecting them from oxidative stress [22,23]. Our analysis reveals that monocytes in the cervical tissue significantly decreased while the MatureDCs and cDC2 increased in PBMC. MatureDCs upregulated T cell activation, MHC II complexes, negative regulation of white blood cell apoptosis γ-Interferon response and other related pathways, suggesting enhanced the immune response ability of T cells [20] as well as γ- The interferon response pathway [24,25]. ClassicaMono-2 and ClassicaMono-3 was the mominant cell in the cervical tissue before treatment while the ClassicaMono-3 cells significantly decreased after treatment. ClassicaMono-3 overexpresses chemokines (CXCL2, CXCL3, CXCL8, CCL4, CCL3L1, CCL4L2, CCL3), while ClassicaMono-4 overexpresses ISG56/IFIT1 family genes (ISG15, IFIT3, IFI6, IFIT2, IFI44L, IFIT1), these genes, stimulated by interferon play multiple regulatory roles in antiviral immunity and interferon signaling pathways [26] (Figure 6).
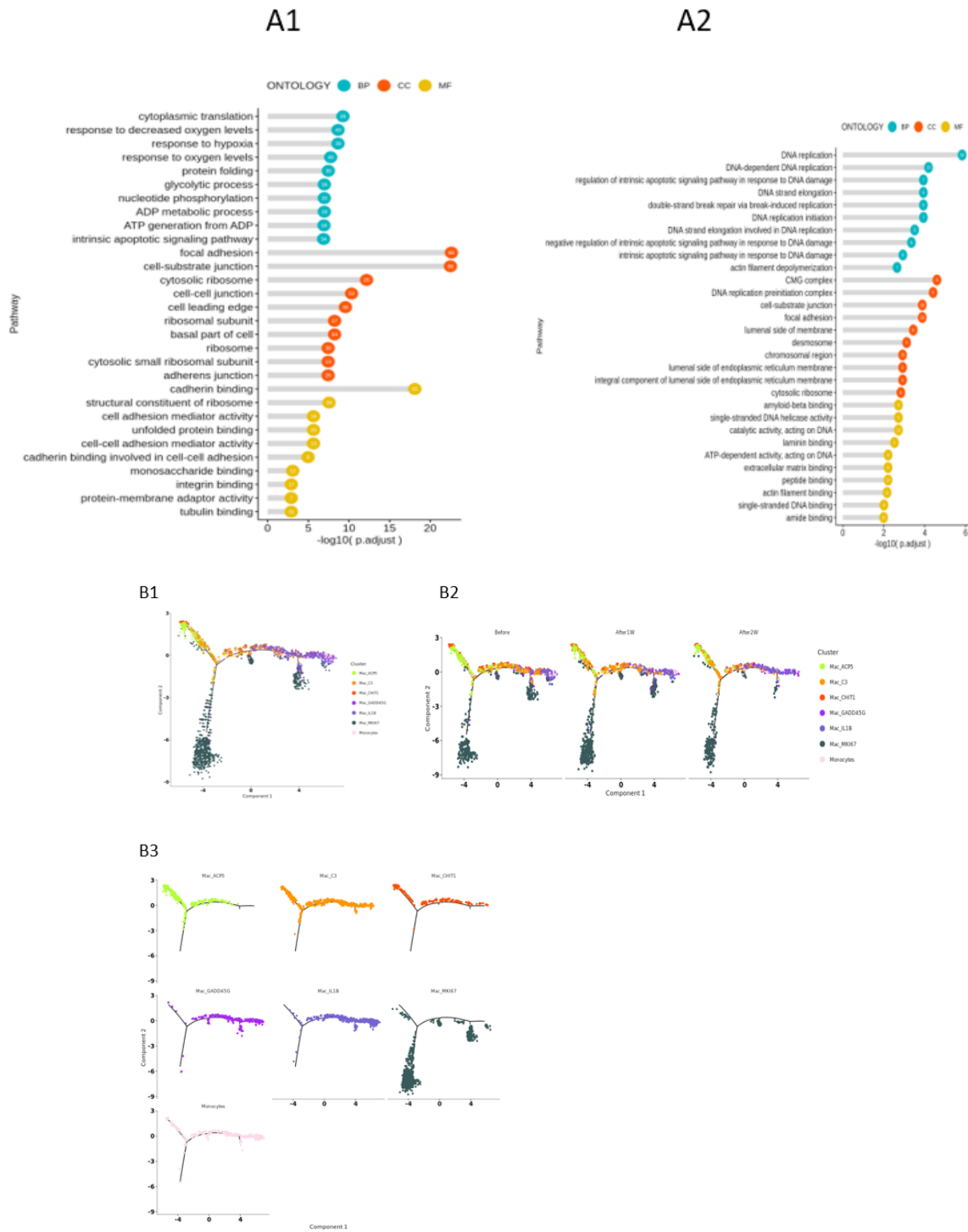
Figure 6: A1; After 2W vs Before CancerCells_ERO1A GO enrichment; A2: After 2W vs Before CancerCells_TOP2A GO enrichment; Quasi-temporal analysis of macrophage subtype monocle

Treatment decreased inflammatory chemokines such as CXCL2, CCL3, CCL4, and CXCL3, especially CXCL8 in the ClassicaMono-2 subgroup, while RFX1 (Regulatory Factor X1) transcription factor is specifically over-expressed in the ClassicaMono-2 subgroup. Some target genes of RFX1 are known to include MHC class II genes, which encode important antigen-presenting molecules in the immune system and participate in processes such as antibody mediated immune responses [27] which is not only for cancer immune reaction and also for HPV immune reaction. Topical application of 2,4-Dinitrochlorobenzene (DNCB) as hapten was employed in the immunotherapy of HPV-associated lesions. It was previously found that hapten of DNCB treatment of skin expressing HPV16. E7 protein, the major oncogenic protein expressed in HPV-associated premalignant cervical epithelium, results in a hyperinflammatory response, with an associated induction of Th2 cytokines and infiltration of myeloid cells producing arginase-1, which also contributes to the hyperinflammation [11].

How does this treatment compare to laparoscopic radical hysterectomy and open approach? Due to laparoscopic radical hysterectomy and open approach treatment of cervical precancerous lesions, positive endometrial margin is a major risk factor for predicting 5-year recurrence [18]. At high risk for having a positive surgical margin and experiencing HPV persistence, positive at the inner margin of the cervix and positive at the outer margin of the cervix (HR: 6.44 (95%CI: 2.80, 9.65); P < 0.001) was associated with an increased risk of persistence/recurrence. By multivariate analysis, only the inner margin of the cervix was positive while the outer margin of the cervix was positive (HR: 4.56 (95%CI: 1.23, 7.95); P = 0.021) was associated with a poorer prognosis. In this high-risk population, a positive cervical margin was the main risk factor for predicting 5-year recurrence [28]. This study demonstrates the important role of awakening immune cells in combating HPV-positive cervical precancerous lesions, restoring normal HPV-negative and abnormal proliferation, and preventing HPV recovery which may be a long-term benefit for the high-risk people with a positive endocervical margin and experiencing HPV persistence and "low-risk" early-stage cervical cancer [29]. It is suggested that immunotherapy should be advanced before or during any treatment to wake up immune cells to recognize tumors or HPV by local administration of drugs and hapten, and PD1 or PD-L1 can be added after waking up immune cells if the patient needs. The results presented in our current study have never been demonstrated earlier by single therapy can induce immune response like immunotherapy. Previous studies have reported that the HAVCR2 encoded protein belongs to the immunoglobulin superfamily and TIM family. This gene affects different types of T lymphocytes in the human body and participates in various immune responses, especially in tumor treatment. HAVCR2 can affect tumor growth by regulating T cell activity and infiltration, indicating enhanced immune signal response in PBMC for whole body immune response.

A significant limitation of the study is the sample size: samples from only on patient were analyzed. Given the significant cost associated with scRNA-seq, we will seek additional funding support to extend our study to more patient samples. Our study provides evidence supporting that hapten mediated local chemotherapy is safe and effective method while it induces a systematic immunity against both cancer cells and HPV by initiating immune response from the precancerous cervical lesions to achieve desirable clinical outcome, which may create a new field of medicine and may be called an chemical immune conization.

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