FAP-targeted radioligand therapy with $^{68}Ga/^{177}Lu$-DOTA-2P(FAPI)$_2$ enhance immunogenicity and synergize with PD-L1 inhibitors for improved antitumor efficacy

SUPPLEMENTARY MATERIALS

MATERIALS AND METHODS

Cell Culture and *in Vitro* Evaluation

The CT26 cell line was purchased from the Chinese National Infrastructure of Cell Line Resource. This cell was cultured in DMEM medium (C11995500BT, Gibco) containing 10% fetal bovine serum (S-FBS-SA-015, SERANA) and 1% penicillin-streptomycin solution (SV30010, HyClone) at 37°C with 5% CO_2. The transfer plasmid encoding mouse FAP CDS (NM_007986.3) was generated by General Bio (Anhui) Co.,Ltd. The cell line CT26 stably transfected with mouse FAP (CT26-FAP) were used for *in vivo* and *in vitro* experiments.

Western blotting analysis

Cells were lysed on ice using RIPA cell lysis buffer containing 1% protease inhibitor and phosphatase inhibitor. Total protein quantification was performed using the BCA protein assay kit. Approximately 20 μg of protein was loaded onto a 10% Bis-Tris polyacrylamide gel, electrophoresed at 80 V, and then transferred onto a PVDF membrane. After blocking with 5% BSA, the membranes were incubated overnight at 4°C with antibodies against FAP (A6349, Abclonal) and β-actin (AC026, Abclonal), followed by incubation with fluorescent secondary antibodies at room temperature for 1 h. Finally, after the addition of ECL to the PVDF membrane, the target bands were observed with a chemiluminescence detection system (CLINX, ChemiScope 6200).

Flow cytometry and immunofluorescence

For flow cytometry experiments, CT26-FAP cells incubated with 3.7 MBq/mL ^{68}Ga-DOTA-2P(FAPI)$_2$ and ^{177}Lu-DOTA-2P(FAPI)$_2$ respectively, for 4 h and 24 h and then replaced with serum-free medium. The cells were resuspended in PBS at a concentration of $1x10^6$ cells/100 μL and incubated with purified anti-mouse CD16/CD32 mAb (#553141, BD Pharmingen™) at 4°C for 5 min to block FC receptors. After that, the PD-L1 flow cytometry antibody (#564716, BD Pharmingen™) was added and incubated at 4°C for 30 min. The cells were washed with 500 μl PBS once and analyzed by flow cytometry (BD FACSCanto II). An isotype control antibody (#562965, BD Pharmingen™) and untreated cells were included as controls. A total of 10,000 cells were collected for each sample.

For immunofluorescence staining of PD-L1 and γH2AX, CT26-FAP cells were seeded in confocal dishes. The CT26-FAP cells were incubated with 3.7 MBq/mL

^{68}Ga-DOTA-2P(FAPI)$_2$ and ^{177}Lu-DOTA-2P(FAPI)$_2$ for 4 h or 24 h, respectively and then replaced with serum-free medium. Each sample was fixed with of 4% paraformaldehyde for 10 min. After that, the cells were washed three times with PBS and incubated with 10% goat serum for 30 min to reduce nonspecific binding. Cells were stained with the anti-PD-L1 antibody (ab213524, Abcam)、anti-γH2AX antibody (CST#9718) overnight, its concentration was set to 1/200, then rinsed three times with PBS. After cells were stained for 1 h with secondary antibody (Abclonal, AS039) and washed three times with PBS, cell nuclei were stained blue with DAPI (UE, D4080).

Regarding the ATP luminescent cell viability assay, CT26-FAP cells were first seeded in a 96-well plate at 100 μL per well, approximately 5000 cells, and then co-cultured with pre-stimulated neutrophils at a ratio of 1:10 for 24 hours. The next day, the culture medium was discarded, and 100 μL of detection reagent was added (Yeasen, 40210ES60). The mixture was shaken at room temperature for 2 min to facilitate cell lysis, then left at room temperature for 10 min before measuring chemiluminescence using a multifunctional microplate reader at a wavelength of 560 nm, with a detection time of 0.5 s per well. Relative cell viability was calculated based on the chemiluminescence readings.

For the Calcein AM /PI live/dead cell staining assay, CT26-FAP cells were seeded in a confocal dish, then co-cultured with pre-stimulated neutrophils at a ratio of 1:10 for 24 h. After removing the culture medium, PBS buffer was added for washing, followed by the addition of 100 μL of pre-prepared staining solution (Lablead, C30002), which was incubated at 37°C for 15 min. Finally, observation was conducted under a confocal microscope.

Isolating single cells from mouse tumor tissues

First, tumor tissues from mice of different treatment groups were collected and rinsed with pre-cooled PBS to remove impurities. The tissues were then minced and digested using 0.5 mg/mL digestive enzyme I in a water bath at 37°C for 30 min. After digestion, the mixture was centrifuged and the supernatant was collected. The remaining precipitate was subjected to a second round of digestion using 0.5 mg/mL digestive enzyme I with a processing time of 30 min. The two rounds of digestion totaled 60 min, and then the supernatant and precipitate were combined and filtered using a 70 μM cytosolic filter (352350, BD Falcon). The mixture was centrifuged at 500 g for 5 min. The precipitate was digested with 1 mL of tryptic protein for 15 min, and then the supernatant was collected and filtered using a 35 μM cytofilter (352235, BD Falcon). The cells were centrifuged again at 500 g for 5 min, and then the cell pellet was resuspended in PBS containing 0.01% BSA.

scRNA-seq Data Processing

FASTQ files from single-cell RNA sequencing (scRNA-seq) were processed through cellcosmo, and genomic comparisons were performed using mm10 (mouse) to obtain 10K expression matrix files for each sample. Subsequently, the 10K matrix file for

each sample was further analyzed by the Seurat package (version 4.3.0) in R software (version 4.2.2). Three quality control (QC) measures were applied to each cell to exclude cells that met any of the following criteria:(1) < 500 expressed genes, (2) proportion of mitochondrial genes > 10%, (3) total number of mRNA molecules < 1000 or > 20,000.

Sample Integration, Dimensionality Reduction, and Clustering

Gene expression matrix was normalized by NormalizeData function using parameters scale.factor = 10000 and normalization.method = "LogNormalize". Top1000 highly variable genes were selected for principal component analysis (PCA) using FindVariableFeatures function and the top20 principal components (PCs) were selected for calculation in FindNeighbors function. Cell clustering was performed by the FindClusters function in Seurat, setting the resolution parameter to 0.5 for all cells and 1 for subpopulations.Uniform Manifold Approximation and Projection (UMAP) was performed using the RunUMAP function with default parameters for 2D visualization. Typical markers for the different cell lineages were obtained using CellMarker [CellMarker (xbio.top)] and these markers were used to assign major cell lineages to each cell cluster.

RNA Velocity Analysis

Initially, we utilized the velocity software to generate a loom file based on the sorted bam file and the genome annotation file 'GRCmm10.gtf'. Subsequently, we merged the loom files for every sample into a single loom file. Next, we utilized the R package sceasy to convert the cell Seurat files of each subpopulation to h5ad files and we employed the Python package scanpy to create the AnnData object using the scVelo algorithm. Finally, we used the Python package scVelo for RNA velocity analysis, calculation of RNA velocity, and visualization of the results.

Monocle2

We utilized Monocle2 (version 2.14.0) to deduce the cellular lineage trajectory of neutrophils based on the top 1000 characterized genes with q-values < 0.001, which were calculated using the DifferentialGeneTest function. Following downscaling and cell sorting, Monocle's default parameters were employed to infer differentiation trajectories.

Analysis of Cell-to-Cell Interactions

We utilized CellChatDB.mouse in CellChat (version 1.6.1) to deduce intercellular interactions between distinct cell types. This method infers the strength of potential interactions between two subpopulations of cells based on gene expression levels. We utilized the netVisual_bubble function to determine receptor-ligand interactions.

SCENIC Analysis

We analyzed transcription factors in each tumor cell using pySCENIC software with the raw count matrix as input. Co-expression networks were calculated using runGenie3, and regulators were identified through RcisTarget analysis. The regulator activity of each cell was scored by AUCell.

Supplemental Figures

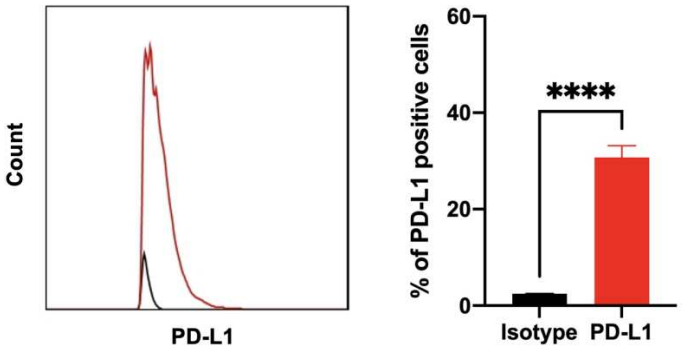

Figure S1. Flow cytometry confirmed PD-L1 expression on untreated CT26-FAP tumor cells. An isotype control antibody served as a negative control to validate specificity (n=3/group).

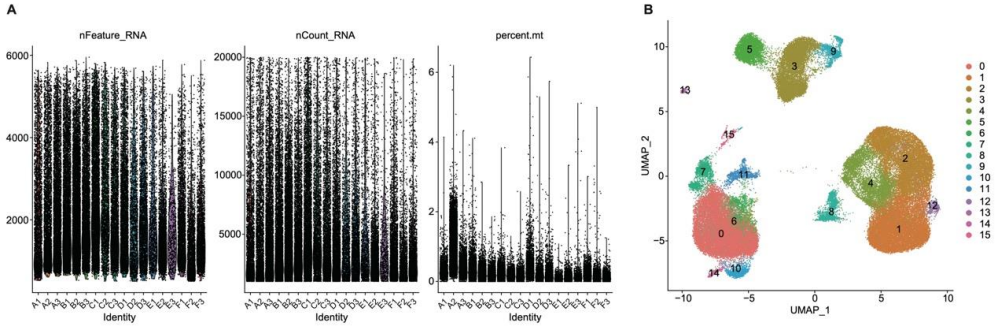

Figure S2. Sample quality control for single-cell sequencing, major lineage clustering, and transcription factor analysis. (A) QC results for single-cell sequencing samples. (B) UMAP plot of all cells categorized into 16 clusters.

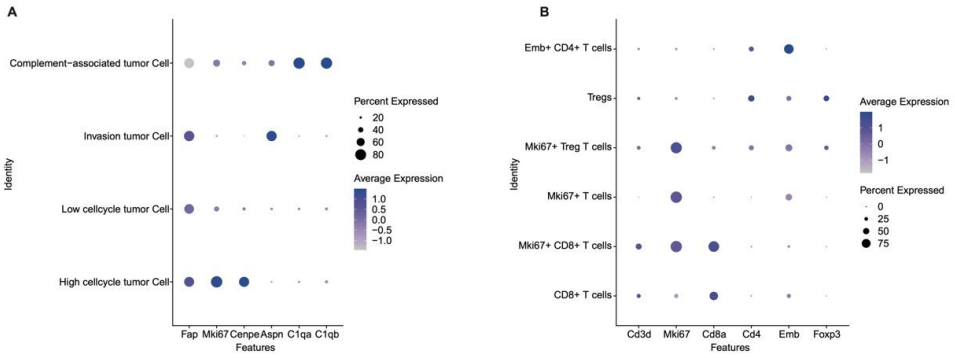

Figure S3. Dot plots reveal characteristic markergene across different tumor cell (A) and T cell (B) fractions.

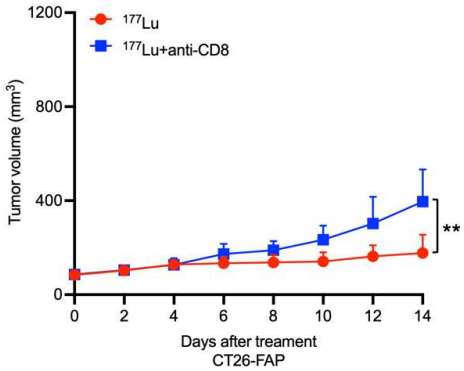

Figure S4. Tumor growth curves of mice treated with ^{177}Lu-DOTA-2P(FAPI)$_2$ alone and in combination with CD8+ T cell blockade (n=6/group).

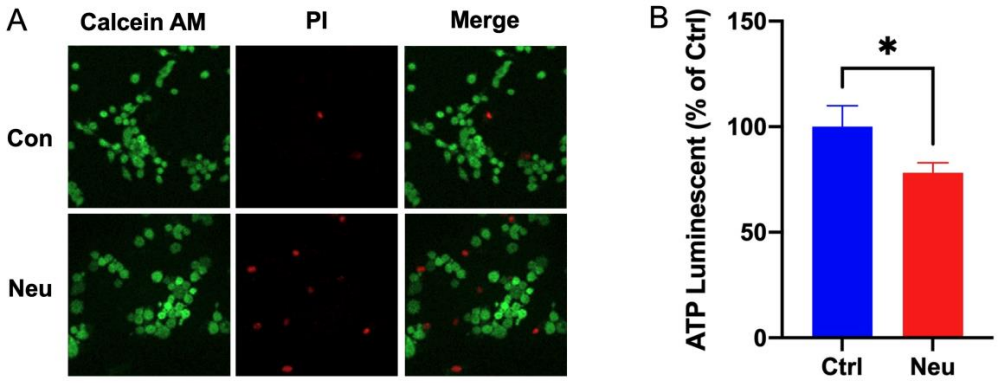

Figure S5. Inhibitory effect of neutrophils on CT26-FAP cell viability. (A) Calcein AM/PI Live-Dead Cell Staining was used to assess the viability of CT26-FAP cells co-cultured with neutrophils, where green indicates live cells and red indicates dead cells (n=3/group). (B) ATP Luminescent Cell Viability Assay was employed to evaluate the viability of CT26-FAP cells co-cultured with neutrophils (n=3/group).

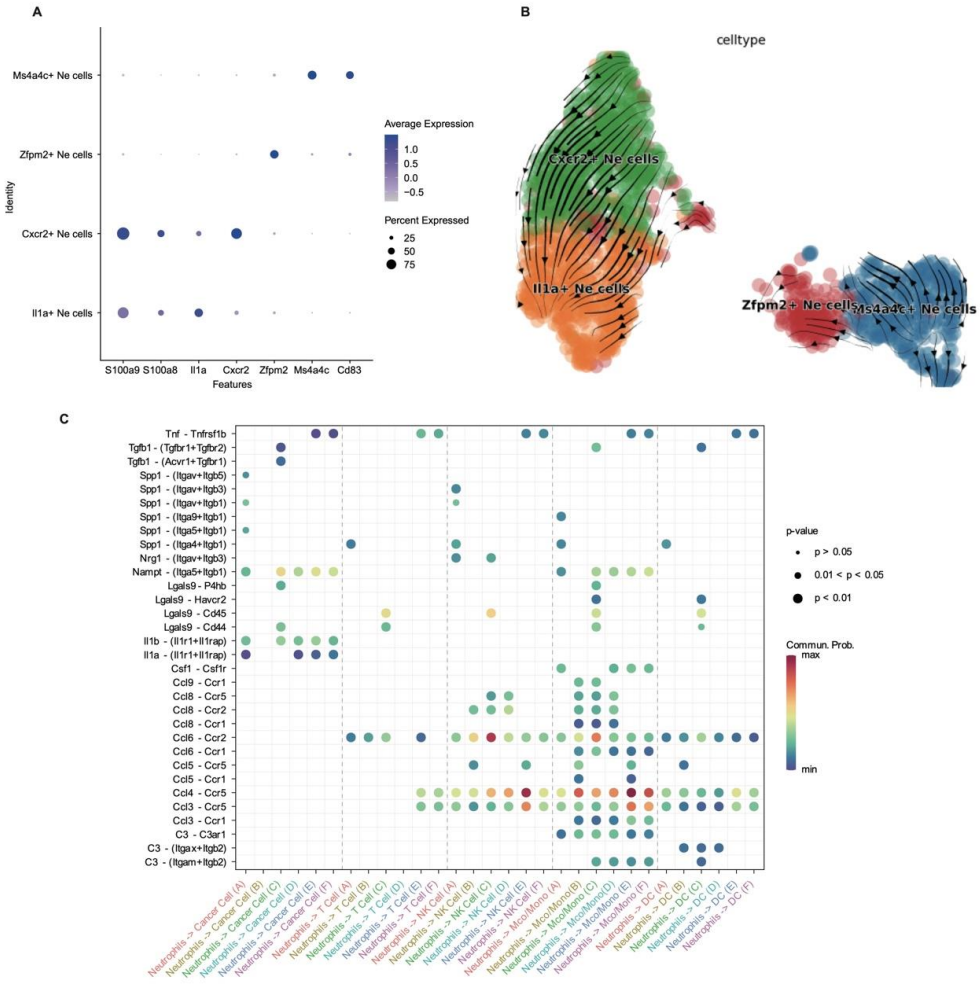

Figure S6. (A) Dot plots reveal characteristic markergene across different neutrophil cell fractions. (B) RNA trajectory analysis reflects the evolutionary process of neutrophil cells. (C) Highlighted ligand-receptor interactions from neutrophil cell subgroups to cancer cells, T cells, NK cells, CAFs, Mco/Mono and DC, as informed by CellChat.