Tissue dissociation

Collected fresh IT or PT samples were washed three times with DPBS containing 10% penicillin G-streptomycin sulfate before being minced into 1-mm3 pieces with a scalpel. The specimens were then digested in X-VIVO 15 medium (Lonza) supplemented with 0.2% collagenase type IV, 0.01% hyaluronidase and 0.002% DNase I (Sigma–Aldrich) for 2 hours at 37°C and overnight at 4°C in 5% CO2. Then, the single-cell suspensions were filtered through a 100 μm strainer and washed twice with DPBS. Leukocytes from peripheral blood were isolated with red blood cells lysis solution (Beyotime Biotechnology)).

***Ex vivo* treatment assay**

Isolated single cells derived from IT tissue were cultured in X-VIVO 15 medium (Lonza) supplemented with 2% human serum (Biological Industries) and 100 μg/ml primocin (InvivoGen) at 37°C and 5% CO2. The cells were treated with pembrolizumab (MedChemExpress, 20 μg/ml), αCD96 (Biolegend, clone NK92.39, 20 μg/ml) or a combination for 24 hours and then subjected to phenotypic analysis by flow cytometry. To assess the ability of the PD-1 blockade to regulate PD-1 expression, αPD-1 (Biolegend, clone NAT105, 20 μg/ml) was used.

Cell culture

The TC-1 cell line was purchased from the Chinese Academy of Sciences Shanghai (CAS) cellular library. The cells maintained in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 100 IU/mL penicillin G, 100 mg/mL streptomycin sulfate, and 10% fetal bovine serum (Thermo Fisher Scientific).

In vivo murine tumor models

C57BL/6 mice (female, 5-6 weeks) were purchased from the Laboratory Animal Center of the Shanghai Institutes for Biological Sciences, and housed in a pathogen-free environment. For establishment of ectopic tumors, 5×10^4 TC-1 cells (in 200 µl of phosphate buffered saline) were injected subcutaneously into the right shoulders of C57BL/6 mice (denoted as Day 0). When the tumor size reached approximately 100 mm^3, the tumor-bearing mice were randomly divided into four groups (Day 12). For PD-1 blockade or CD96 blockade, transplant recipients received 200 µg of αPD-1 (Biolegend, clone RMP1-14), 200 µg of αCD96 (Biolegend, clone 3.3) or a combination twice a week × 4 doses (Day 12, Day 16, Day19, Day23). For the control group, mice received IgG control (Biolegend) at 200 µg/mouse on a similar schedule. For immune response analysis, tumors were excised on day 26. Tumor tissues were digested in RPMI 1640 medium supplemented with 0.2% collagenase type IV, 0.01% hyaluronidase and 0.002% DNase I for 3 hours at 37°C in 5% CO2, and the TILs were separated with Ficoll (Dakewe Biotech Company) followed by flow cytometry analysis. Tumor size was monitored every 3-4 days, and the survival of the mice was determined by denoting the last day of ethical tumor size measurement as the time of sacrifice.

Flow cytometry

Single-cell suspensions of blood and PT/IT samples were washed twice with PBS and incubated for 30 min in the dark with antibodies recognizing specific surface proteins. To analyze cell apoptosis, an annexin V apoptosis detection kit with propidium iodide (PI; BioLegend) was

used. For the detection of intracellular proteins, cells were fixed and permeabilized using a fixation/permeabilization wash buffer (BioLegend) and stained with antibodies for 30 min in the dark. For Ki67 staining, the cells were incubated in 70% ethanol for 1 hour at -20°C and then stained with an anti-Ki67 antibody (BioLegend). All samples were run on a CytoFLEX platform (Beckman Coulter) and analyzed using FlowJo version 10.8 software (BD Biosciences).

Immunohistochemistry (IHC) and immunofluorescence (IF)

Human and mouse tumor tissue samples were fixed in paraformaldehyde and embedded in paraffin wax. Tissue samples were cut into 5-μm-thick slices and baked overnight in a 60 ℃ dehydration oven. The slides were deparaffinized and rehydrated, and the antigens were retrieved by boiling in sodium citrate. Sections were blocked with goat serum and then stained with CD155 antibody (Abcam, ab267788, 1:500), CD111 antibody (Abcam, ab279364, 1:150), or CD112 antibody (Abcam, ab233384, 1:250) followed by a secondary antibody (Servicebio, GB23303, GB23301, 1:200). The procedure was performend with a 3,3'-diaminobenzidine (DAB) kit (Mxb Bio). The negative control was set up in parallel. IF staining was performed with CD8 antibody (Proteintech, 66868, 1:400), CD96 antibody (abcam, ab264416, 1:200) CD155 antibody (Abcam, ab267788, 1:300), CD111 antibody (Abcam, ab279364, 1:150), or CD112 antibody (Abcam, ab233384, 1:200) followed by conjugated secondary antibody (Servicebio, GB21303, GB21301, GB25303, 1:300,).