Supplementary Methods

"Sandwich" cell culture

Place on ice the following:
Collagen type I (cons: 8.43 mg/ml)
Sterile 10X DMEM
Sterile ddH20
Sterile 0.023N NaOH
For 24 well plate, the volume of collagen solution is 100 ul with 1.5 mg/ml (lower) or 1.0 mg/ml (upper).

Lower (1.5 mg/ml):
If the final volume of collagen solution is 1000 ul, 1000*1.5/8.43 = 178 ul; 178 ul collagen type I is to add.
The volume of NaOH is same as collagen. 10X DMEM is one tenth of total volume.
H2O volume = (final volume)-(volume collagen)-(volume NaOH)-(10X DMEM)
Collagen-178 ul
10X DMEM-100 ul
NaOH-178 ul
ddH2O-544 ul

Upper (1.0 mg/ml):
Collagen-119 ul
10X DMEM-100 ul
NaOH-119 ul
ddH2O-662 ul
Mix the contents of tube and hold in ice.

Procedure:
Deliver the collagen solution into well and allow to gel at 37°C for 2 h. Add cells suspension into well, incubate overnight in 37°C.
Wash the cell 2 times with sterile PBS.
Deliver the collagen solution into well and allow to gel 37°C for 2 h. Add 250 ul culture solution per well.

Immunofluorescence staining (2D-12 wells)

Wash the wells with pre-warmed PBS (500 µl per well) 1-2 times.
Fix the samples with 4% paraformaldehyde/PBS pH 7.4 (500 µl per well) for 15 min at room temperature.
Wash samples 3 times with ice cold PBS (500 µl per well), 5 min each.
Proceed with staining or store at 4°C until staining.
Make the Wash Buffer (PBS, 0.1% BSA, and 0.1% Tween) and Blocking Buffer (PBS, 10% NGS/NDS, 1% BSA, 0.1% Tween, and 0.1% Triton X-100) before starting the staining.
Wash the samples 2 times with 500 µl PBS (4°C stock samples).
Wash the samples 3 times with 500 µl Wash Buffer, 5 min each wash.
Block and permeabilize samples for 1 hr with blocking Buffer.
Aspirate blocking buffer and apply Primary antibody diluted in blocking buffer: COX-2-1:200, and incubate overnight at 4°C.
Wash the samples 3 times with 500 µl Wash buffer, 5 min each wash.
Apply Second antibody diluted in blocking buffer: Donkey anti rabbit Alexa 488-1:250, and incubate 1 hr at room temperature.
Wash the samples 3 times with 500 µl Wash buffer, 5 min each wash.
Wash 2 times with PBS, and incubate samples in 500 µl 1 ug/mL Hoechst 33342 in dark for 1 min. Then wash 3 times with PBS and proceed with imaging. For long-term storage, store the samples in the dark at 4°C.

Immunofluorescence staining (Paraffin section)

A. Dewaxing/rehydration
Note: Make sure to keep the slices moist at all times during the process
1. Turn on the exhaust air to high grade
2. Incubate the slices in xylene solution for 5 minutes and 3 times
3. Incubate the slices in 100% ethanol for 5 minutes
4. Incubate the slices in 95%, 70% and 50% ethanol successively for 5 minutes each time
5. Hydrate with ddH2O for 5 min and put into PBS

B. Antigen repair
1. Transfer the loading sheet to the pressure cooker and add the appropriate antigen-repair buffer to the pressure cooker suitable for microwave oven (see Appendix 1 for recipe).
2. Seal pressure lid according to manufacturer's instructions
3. Turn the microwave on high and time it for 15 minutes
4. After 15 minutes, turn off the microwave oven and cool the pressure cooker at room temperature for 40 min (set up a blocking buffer and see attachment 2 for the formula).

C. Incubate primary antibody
1. Wash the slides with PBS solution for 3 times, 5 min each
2. Add NaBH4 to the new PBS solution with a concentration of 0.1%. Immediately after the bubbles appear, put the slides into PBS solution and incubate the slides at room temperature for 30 min
3. Wash the slides with PBS solution for 3 times, 5min each
4. Suck out the liquid on the tissue, and incubate the sheet with blocking buffer at room temperature for 1 hour
5. Suck the liquid from the tissue, then move the wet box into the refrigerator at 4°C and incubate with the primary antibody (COX2-1:200, CD163-1:200, CD206-1:200)
6. Incubate at 4°C overnight

D. Incubate fluorescent secondary antibody
1. Dilute the fluorophore coupling secondary antibody with blocking Buffer (before absorbing the secondary resistor, shock the vertex secondary resistor for 10 s, then add the secondary resistor to the blocking buffer for dilution, wrap the EP tube or 15 ml centrifuge tube in foil and store at 4°C)
2. Wash the slides in PBS solution three times, 5 min each time (if washed in glass, wrap them in tin foil).
3. Use the vertex shakily diluted secondary antibody for 10 s to absorb the fluid from the tissues and coat the secondary antibody on the tissues
4. Incubate at room temperature for 1 hour
To avoid the photobleaching effect, these operations should be performed in the dark.
5. Wash with PBS solution 3 times, 5 min each time (if washing with glass, wrap with tin foil)
6. Nuclear staining with anti-quenchants with DAPI, cover with cover glass, after exhaust bubble, seal with nail oil, and store at 4°C for photography
The fluorescence staining was generally observed within 1 h, or stored at 4°C for 4 h, which would weaken the fluorescence for too long

Antigen repair solution formulation

Sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0)
- (Dihydrate) 2.94 g trisodium citrate
- Distilled water 1 L
- Mix and dissolve, adjust pH to 6.0 with 1N HCl.
- Add 0.5 ml Tween 20 and mix well. Store at room temperature for 3 months or before storage
- Long-term storage at 4°C

Blocking buffer formula
- 2% NGS or NDS
- 1% BSA
- 0.1% of Triton X-100
- 0.1% Tween
- PBS

Immunohistochemical Staining (Tissue chips)

I. Preparation of reagents
1. The preparation and use of EDTA antigen repair solution are shown in the product instructions.
2. Preparation of AP-red chromogen (pay attention to shake 4B solution well before preparation)
1) Add 200 ul of reagent 4B (GBI-AP-red activator, shake well before use) to 1 ml of reagent 4A (GBI-Ap-red substrate), mix well, then add 10 ul of reagent 4C (GBI-AP-red substrate) to the above mixture, mix well again. (If the amount used is not much at one time, a suitable amount of color developing liquid can be prepared according to the above proportion).
2) Add 50-100 microliters or enough of the above working fluid to cover the tissue to each section, incubate for 10 minutes, and observe the color development under the microscope. To enhance the dyeing effect, the liquid can be prepared fresh again and the process can be repeated.
3) Rinse with distilled water to stop color development
3. Preparation of DAB color developing agent
1) The working liquid of DAB is prepared before use. In 1 ml of reagent 3A (DAB diluent), about 50 microliters of reagent 3B (DAB concentrated liquid) is added and evenly mixed, that is, the working liquid of DAB is prepared. This solution must be used as needed, stored away from light after preparation, and used up within 7 hours.
Note: THE WORKING liquid of DAB needed in the experiment can also be prepared according to the above proportion
2) Add the above DAB working liquid enough to cover tissues, and observe the color development under the microscope, which takes about 5-8 minutes
3) Rinse with distilled water to terminate the reaction. TBS-T rinse, 2 min × 3 times

II. Experimental steps
1. Paraffin section, dewaxing to water.
2. Tbs-t buffer was cleaned for 3 times, 2 min/each time.
3. If necessary, the tissue sections should be repaired with antigens according to the primary antibody repair method provided by the manufacturer.
High pressure repair may refer to the following: preheat repair fluid pressure cooker, after boiling will be placed in the plastics dyeing glass on the shelf in the repair of liquid, must be completely covered, timing starts when the pressure limiting valve rotating jet 2.5 min, timing and induction cooker from high heat to medium, timing to leave after the heat source, cold water shower to room temperature, be careful not to cold water into a pressure cooker.
4. Tbs-t buffer was cleaned for 3-5 times, 2 min/time.
5. Add an appropriate amount of endogenous peroxidase blocker (H2O2) and incubate at room temperature for 10 min.
6. Clean the distilled water and immunohistochemical stroke circle (2~3 mm away from the tissue when drawing the circle).
7. Tbs-t buffer was cleaned for 3-5 times, 2 min/time.
8. Add an appropriate amount of the mixture of primary antibody, 37°C for 1 hour (when adding primary antibody, the water on the slices should be shaken clean, and do not dry the slices, COX2-1:200, CD163-1:200; CD206-1:200).
9. Tbs-t buffer was cleaned for 3-5 times, 2 min/time.
10. Add an appropriate amount of mixed secondary antibody at 37°C for 30 min.
11. Tbs-t buffer was cleaned for 3-5 times, 2 min/time.
12. Add chromogenic agent A for 10-15 min, and the positive color is red.

13. Tbs-t buffer was cleaned for 3-5 times, 2 min/time.
14. Add color developing agent

Coculture system in vitro

$CD8^+$ T cells were divided into four groups: one group was cocultured with the hepatocellular carcinoma cell lines and $CD14^+$ monocytes for 24 hours; one group was cocultured with hepatocellular carcinoma cell lines alone for 24 hours; one group was cocultured only with $CD14^+$ monocytes (induced by hepatocellular carcinoma cell lines) for 24 hours (Supplementary material-Figure S1).

During the whole co-culture process, the co-culture system contained 0.5 million hepatocellular carcinoma lines, 0.1 million primary $CD14^+$ monocytes, and 0.1 million primary $CD8^+$ T cells. In the process of exploring the conditions, we also tried to put 0.5 million primary $CD8^+$ T cells in the co-culture system, but the results were not statistically significant, so we finally adopted the co-culture program of 0.1 million primary $CD8^+$ T cells (Supplementary material-Figure S2).

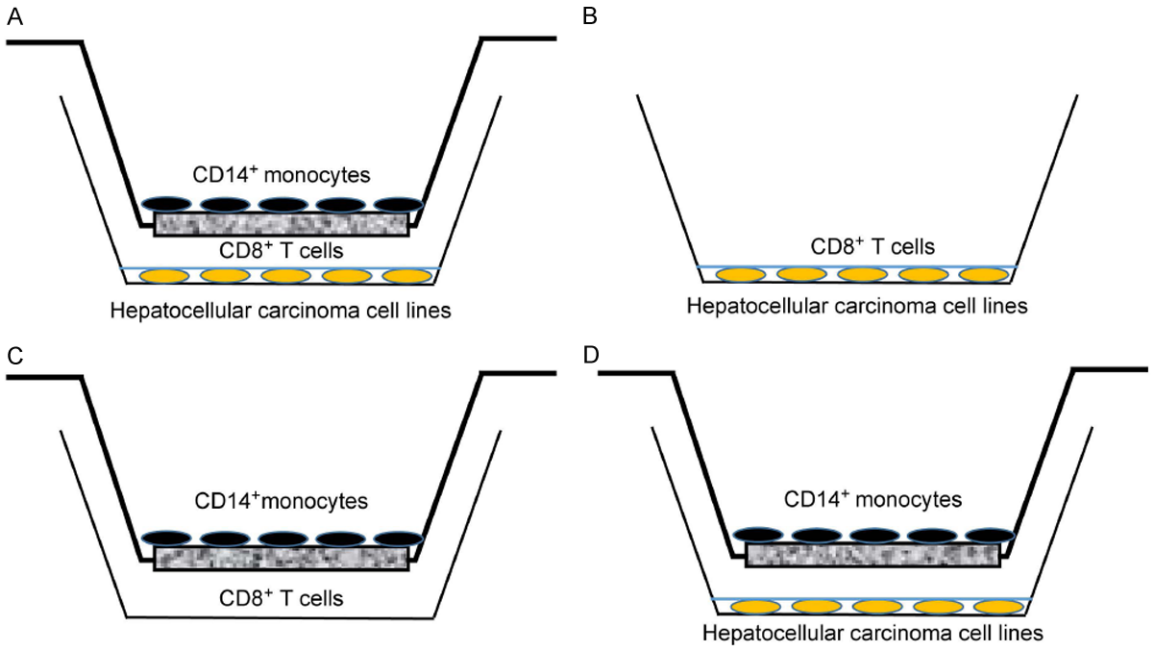


Figure S1. In vitro co-culture model.

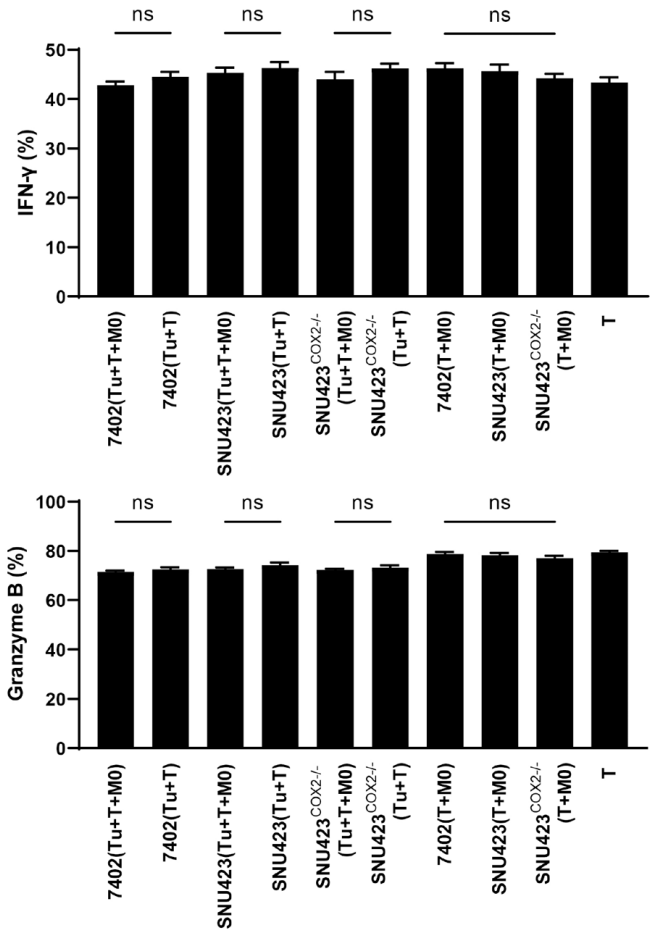
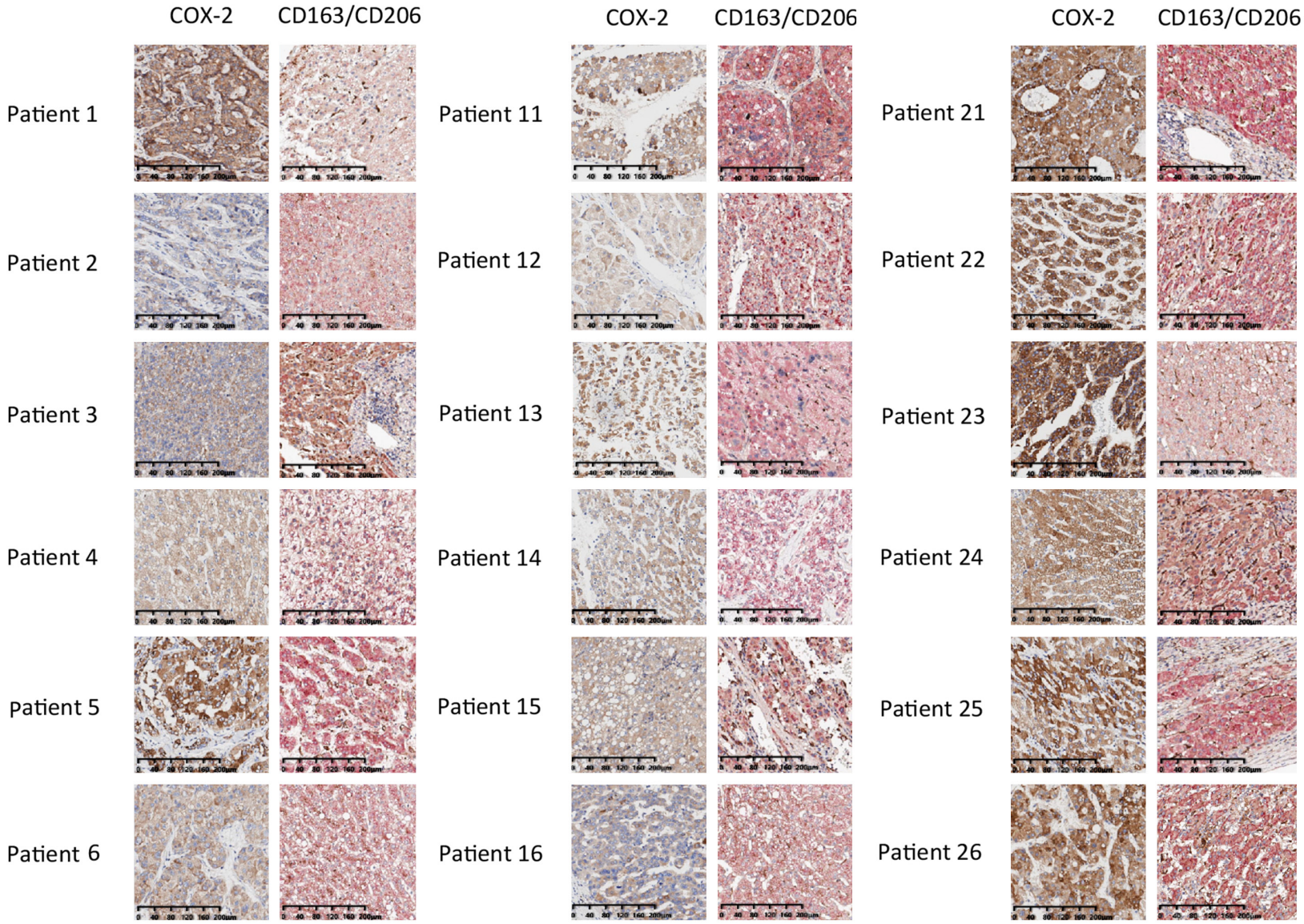


Figure S2. Flow cytometry results of 0.5 million CD8+ T cells added into the co-culture system.


COX-2
CD163/CD206
COX-2
CD163/CD206
COX-2
CD163/CD206
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 11
Patient 12
Patient 13
Patient 14
Patient 15
Patient 16
Patient 21
Patient 22
Patient 23
Patient 24
Patient 25
Patient 26

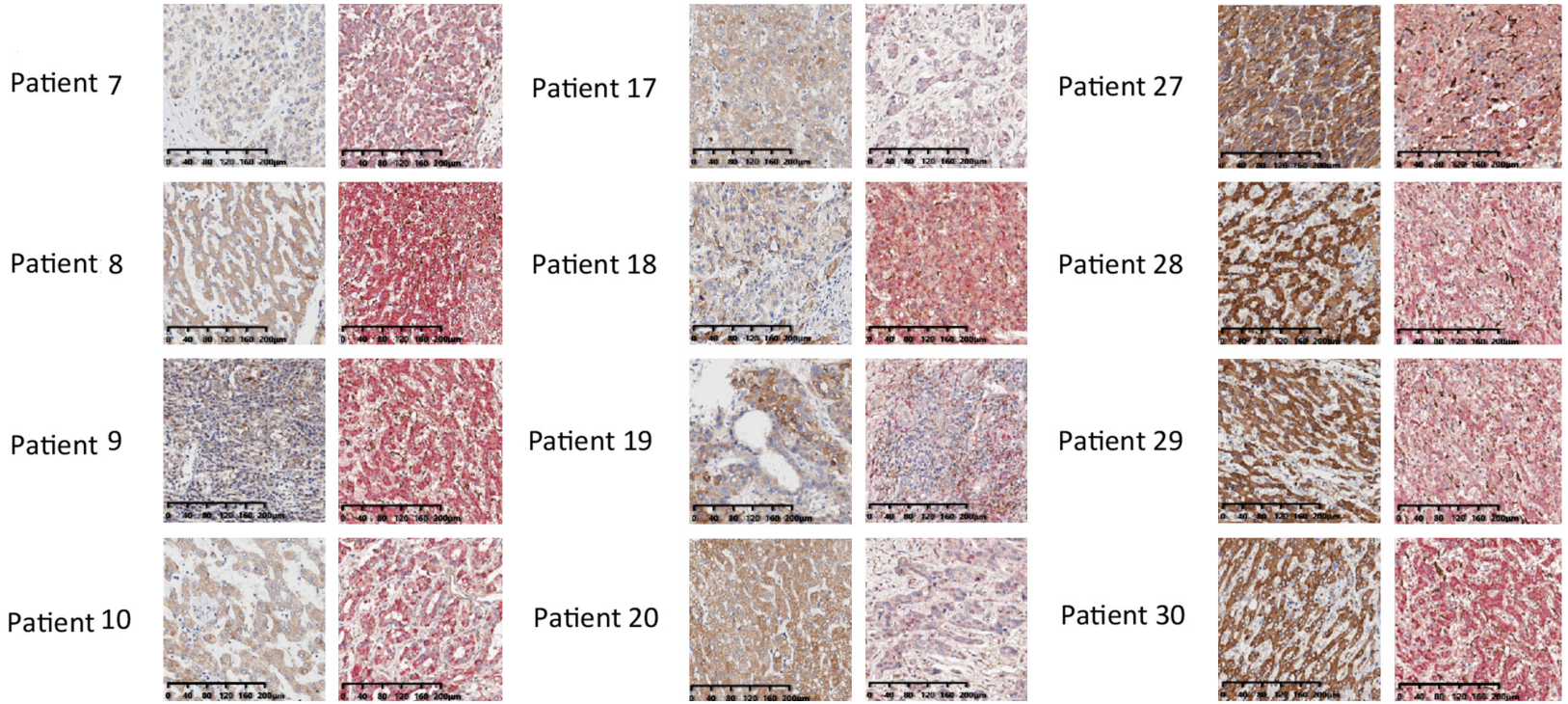


Figure S3. Immunohistochemical staining of tissue microarray from 30 patients with hepatocellular carcinoma.

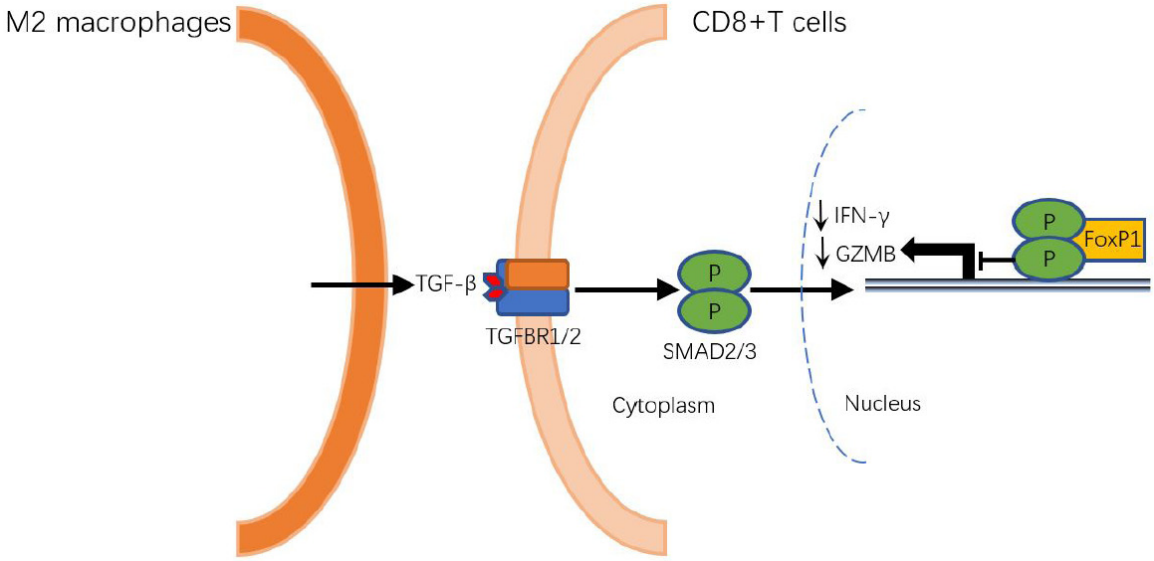


Figure S4. TGF-β signaling pathway diagram.