Supporting Information for

Single-cell Secretion Analysis in the Engineered Tumor Microenvironment Reveals Differential Modulation of Macrophage Immune Responses

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This Supporting information includes: Experimental methods, characterization of PDMS micropillars, perforated PDMS microchamber, and the viability of MCF-7 cells and U937-derived macrophages; Secretion analysis for two or three primary monocyte-derived macrophages in engineering primary tumor microenvironment; Information for antibodies used, primary tumor and monocytes.

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Cell culture, differentiation, stimulation, and staining: MCF-7 cell line (American Type Culture Collection, ATCC) was cultured in DMEM/F-12 medium with 10 % FBS (PAN, Germany). Human leukemic monocyte cell line U937 and THP-1 (ATCC) were cultured in RPMI 1640 medium supplied with 10 % FBS (PAN, Germany). The U937 and THP-1 cells were differentiated into macrophages with

50 ng·mL^{-1} PMA (phorbol 12-myristate 13-acetate, Sigma) for 48 hrs. The differentiated cells were cultured with fresh RPMI 1640 complete medium and cultured for another 48 hrs before experiments. Human primary monocytes were isolated from PBMC of healthy donors using a pan monocyte isolation kit (Miltenyi Biotec) and differentiated for 7 days in RPMI medium with 50 ng·mL^{-1} GMCSF (R&D, USA), 20 % heat-inactive FBS. Differentiated macrophages were harvested by 0.02 % EDTA solution in PBS (pH=7.2). The macrophages were treated with 100 ng·mL^{-1} LPS (Sigma, USA) before cell loading for stimulation. The human primary tumor cells were obtained from the Affiliated Hospital of Dalian Medical University. The collection and use of human samples were approved by the Ethics Committee of Dalian Medical University. The human primary tumor cells were cultured with DMEM high glucose medium supplied with 10 % FBS.

After harvested by trypsin-EDTA (0.25 %), tumor cells were centrifugated for 10 mins at 1000 rpm. The supernatant was replaced by 1 mL DMEM serum-free medium. Cancer cells were gently resuspended. Then 5 μL DIL (cell tracker dye, Vybrant) was mixed into the suspension, well mixed, and incubated for 20 mins at 37 ℃. To remove the dye, cancer cells were centrifugated, gently resuspended by serum-free DMEM medium without dye, and centrifugated again. After repeating this washing procedure two times, cancer cells were resuspended and mixed with U937 macrophages stained with Calcein-AM for coculture.

Preparation of antibody-coated glass slide for secretion detection in single-cell and bulk assay: 100 μL solution containing capture antibodies (1:50 dilution in DPBS) was pipetted onto a poly-L-lysine (PLL) glass slide, and a clean cover glass was pressed onto the PLL glass slide slowly to minimize bubble generation during the process. The glass slide was incubated at 4 °Covernight under humidity conditions. After taking off the cover glass, the glass slide was blocked with 3 % BSA for 1h. Then the glass slide was washed with DPBS, 50 % DPBS, DI water, DI water sequentially and dried with an air gun. For the preparation of the antibody-coated glass slide for bulk level secretion detection, a PDMS microchip with parallel microchannels was boned onto the PLL glass slide. Capture antibodies (1:4 dilution in DPBS) were patterned onto the glass slide through respective microchannels, including IL-10, IL-8, IL-6, TNF-a, MCP-1 MIP-1b. After overnight incubation at 4 °C, the microchannels were flushed with 3% BSA solution and blocked for 30 mins. Then, the PDMS microchip was disassembled, and the glass slide was washed and dried for bulk assay.

Assembly of the single-cell analysis platform: The perforated PDMS stencil array was aligned onto the PDMS micropillar substrate, and the integrated chip was sterilized with ultraviolet for 30 mins and treated

with plasma for 2 mins. After plasma treatment, the fresh medium was added to the PDMS to keep the surface hydrophilic. Two PDMS slabs (1 mm thickness,1.5 cm length) was placed at the end of the microchip, in which a sterile glass slide was placed as a cover to facilitate uniform cell loading into microchambers and remove residual cells out of chambers.

Immunofluorescence staining: 200 μL solution containing PE anti-human CD68 (Biolegend) and FITC anti-human CD163 (Biolegend) was added onto the macrophages and incubated for 20 mins at room temperature. After incubation, the cells were washed 3 times with DPBS, fixed with 4 % paraformaldehyde for 15 mins, and permeabilized by 0.1 % Triton X-100 for 20 mins. Then APC anti-human CD206 (Thermo Fisher) and DAPI (100 $ng \cdot mL^{-1}$ in DPBS) solution were added and incubated for another 20 mins. After stringent washing, cells were imaged by the Nikon Eclipse Ti-E and Yokogawa CSU-W1 microscope.

Supporting Information

Supporting Information is available from https://pubs.acs.org.

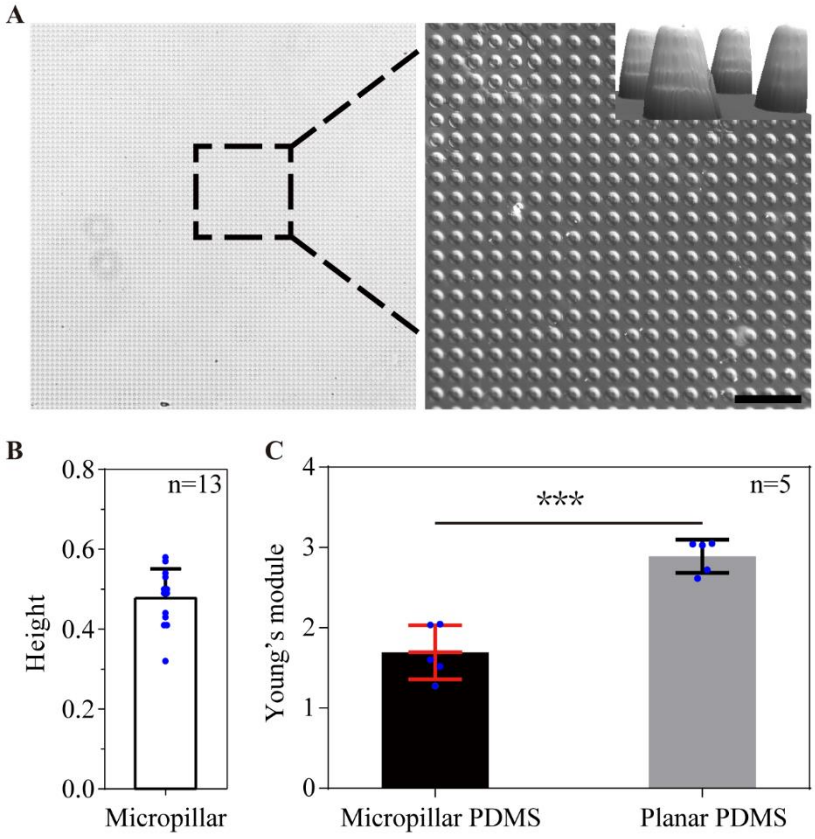

Figure S1. Characterization of PDMS micropillars substrate. A) Representative brightfield image showing the high-density PDMS micropillar array (diameter: 5 μm) fabricated by soft lithography and its enlarge view. The inserted image representing micropillars morphology by AFM. Scale bar: 50 μm. B) Height (surface profiler) characterization; C) Young's module on different substrates (Instron 5567A).***representing P<0.001.

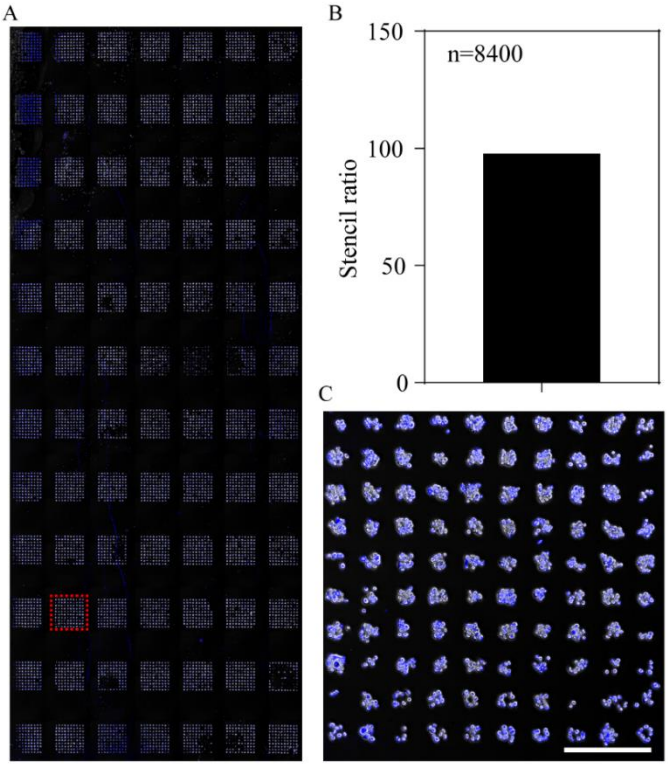

Figure S2. Stencil ratio test by cells seeding method. Large scale characterization of perforated PDMS microchamber array using cells seeding method. The perforated PDMS stencil was attached to a clean glass slide, in which adherent cells were seeded afterward. The cells would fall into the microchambers and adhered to the bottom of the glass slide when the microwell is perforated. We then fixed the cells and stained their nuclei with DAPI after the cells have fully adhered to the substrate. The PDMS layer was peeled off before imaging under the fluorescence microscope to check the occupancy of the cells on the glass slide. A) Whole slide scanning of glass slide after removing PDMS stencil; B) Its enlarged view of red marked area; C) 97.7% (n=8400) of microchambers were entirely perforated. Scale bar: 500 μm.

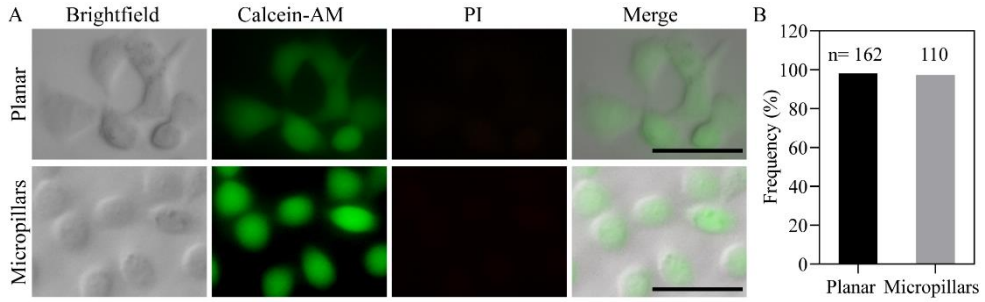

Figure S3. Comparison of MCF-7 cells viability on planar and micropillars PDMS. (A) Representative images of MCF-7 cells cultured on planar and micropillars. MCF-7 cells were stained with Calcein-AM and PI dye after cultured for 24 hrs on different substrates. Scale bar: 50 μm. (B) The viability of MCF-7 cells on planar (98.1%, n=162) and micropillars (97.3%, n=110).

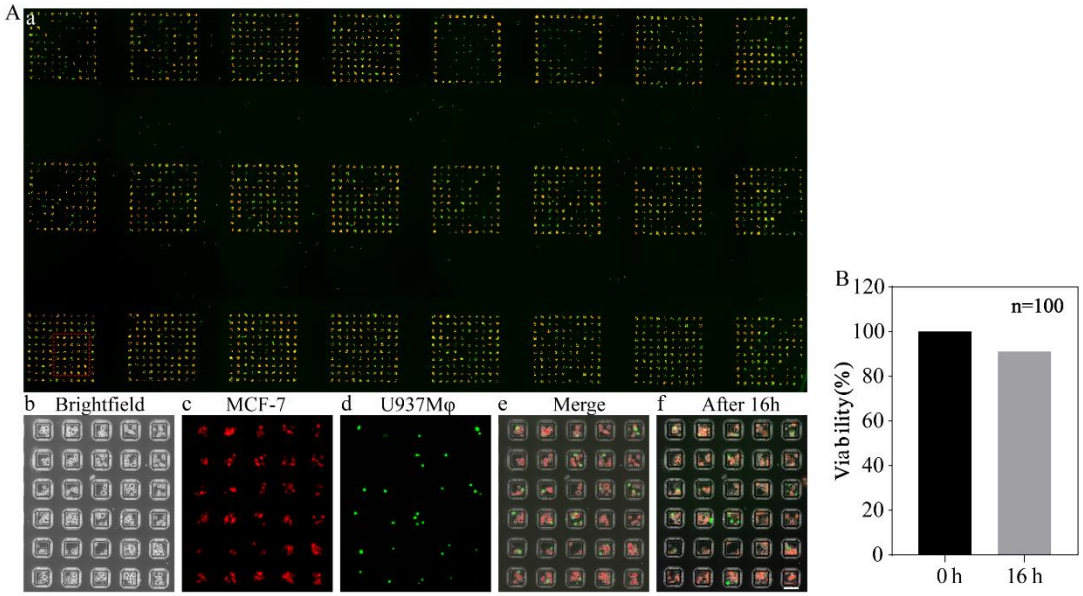

Figure S4. Characterization of engineered tumor microenvironment for single-cell secretion analysis platform. (A) Scanned large image and enlarged images of U937-derived macrophages and MCF-7 cocultured in microchambers with micropillar substrate. Scale bar: 100 μm. (B) Viability test of U937-derived macrophages and MCF-7 cocultured after 16 hrs in microchambers, n=100.

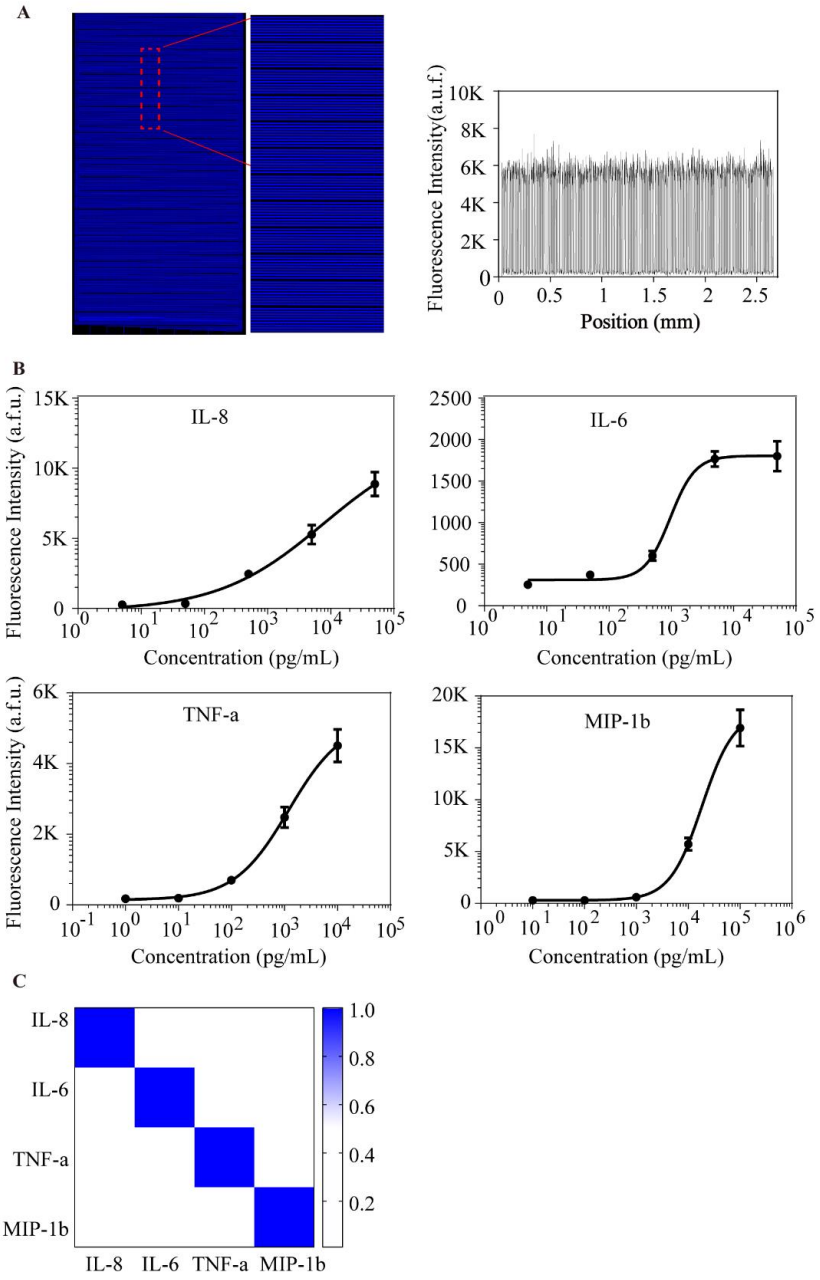

Figure S5. Characterization of protein detection on PLL (Poly-L-lysine) coated glass slide. A) Large area fluorescence image and fluorescence intensity profiles of the marked area showing proteins immobilization uniformity via the patterning of BSA-FITC on to the glass slide. B) Titration curves of antibody pairs with corresponding recombinant proteins. C) The crosstalk test of antibodies pairs with related recombinant proteins. The fluorescence intensities were obtained from the averaged value of 30 spots for each protein. Error bars show 1× SD.

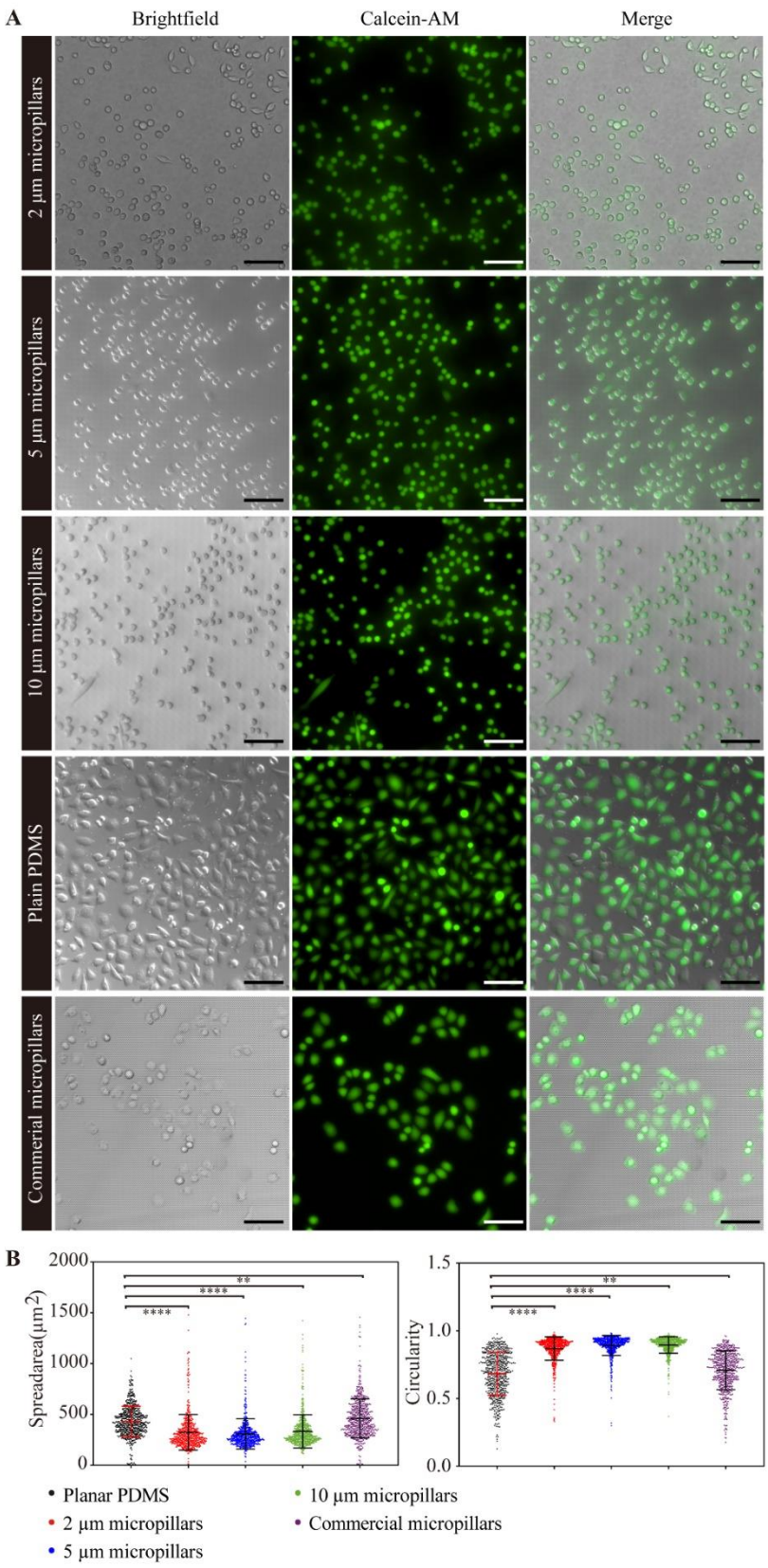

Figure S6. Comparison of the morphological differences on 2D and 3D substrate. A) Representative images showing MCF-7 cells cultured on 2D substrate (planar PDMS) and 3D substrates. Scale bar: 100 μm. B) Statistical results of cell spreading area and circularity on the corresponding substrates. The spread

area of MCF-7 on different substrates was listed: 2D: 431.6 ± 148.8; 2 μm micropillar: 324.1 ± 174.9; 5 μm micropillar: 308.0 ± 148.2; 10 μm micropillar; 334.1 ± 163.7; commercial micropillar: 460.4 ± 191.9, n=600; **P<0.01, ****P<0.0001. Circularity of MCF-7 cells cultured on substrates was separately 2D: 0.68 ± 0.16; 2 μm micropillar: 0.87 ± 0.07; 5 μm micropillar: 0.89 ± 0.07; 10 μm micropillar; 0.90 ± 0.06; commercial micropillar: 0.71 ± 0.14, n=600; **P<0.01, ****P<0.0001.

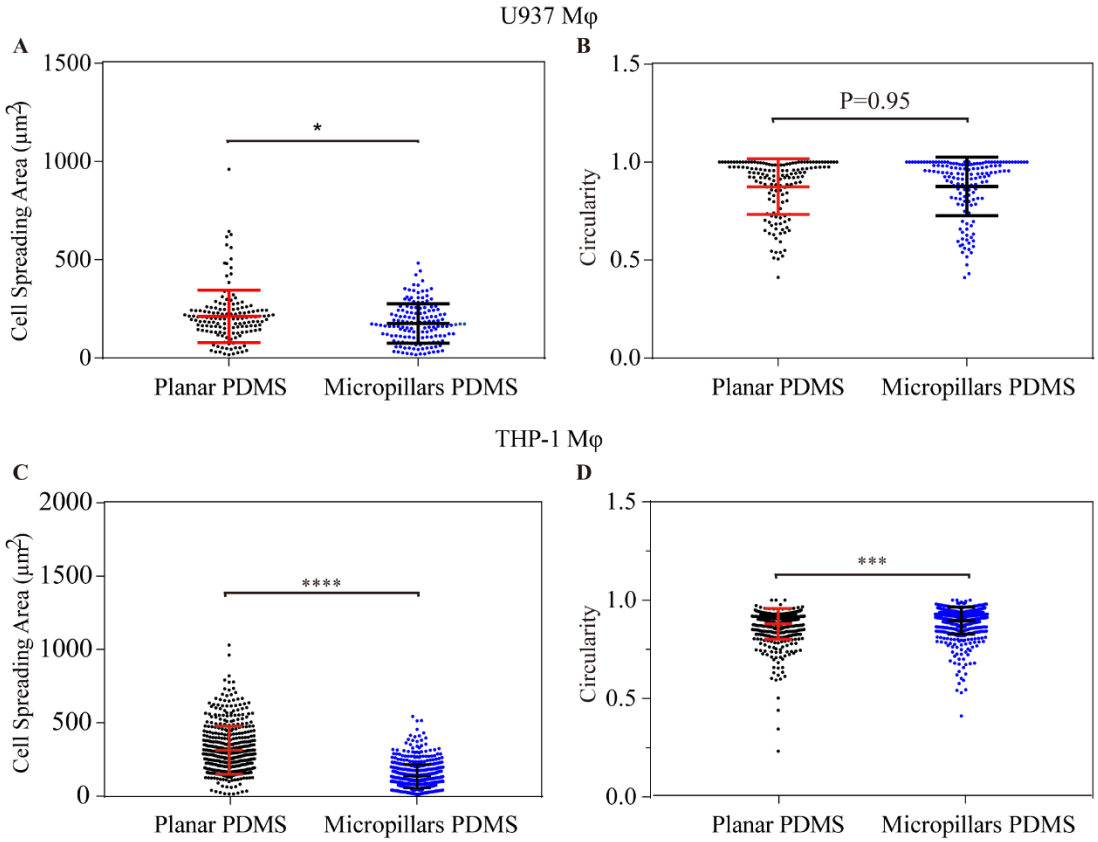

Figure S7. Morphology characterization of macrophage cells cultured on different substrates. A, B) Cell spread area and circularity of U937-derived macrophages on the 2D substrate (planar PDMS) and 3D substrate (5 μm PDMS micropillar). C, D) Cell spread area and circularity of THP-1-derived macrophages on 2D and 3D substrate (* P<0.05, *** P=0.001, ****P<0.0001).

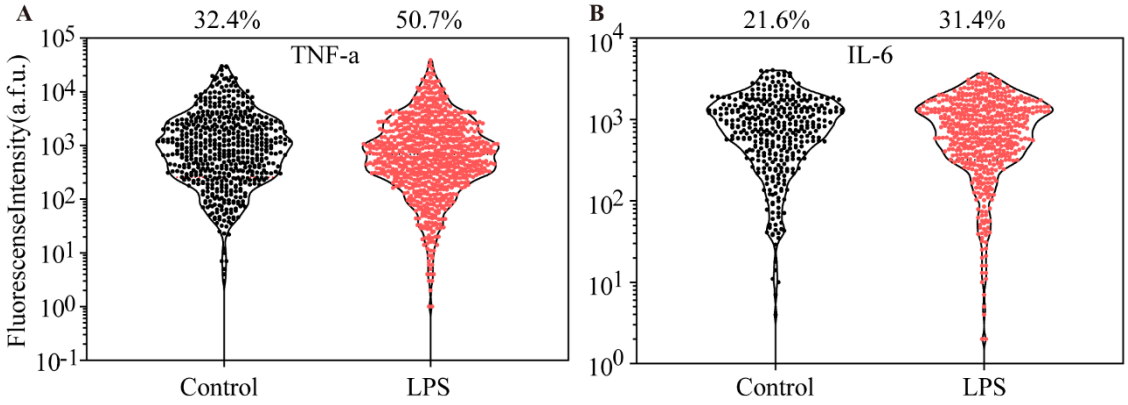

Figure S8. Single-cell secretion analysis in engineering primary tumor microenvironment. A) TNF-a and B) IL-6 secretion from single THP-1-derived macrophages in the basal group (control) or stimulated with LPS.

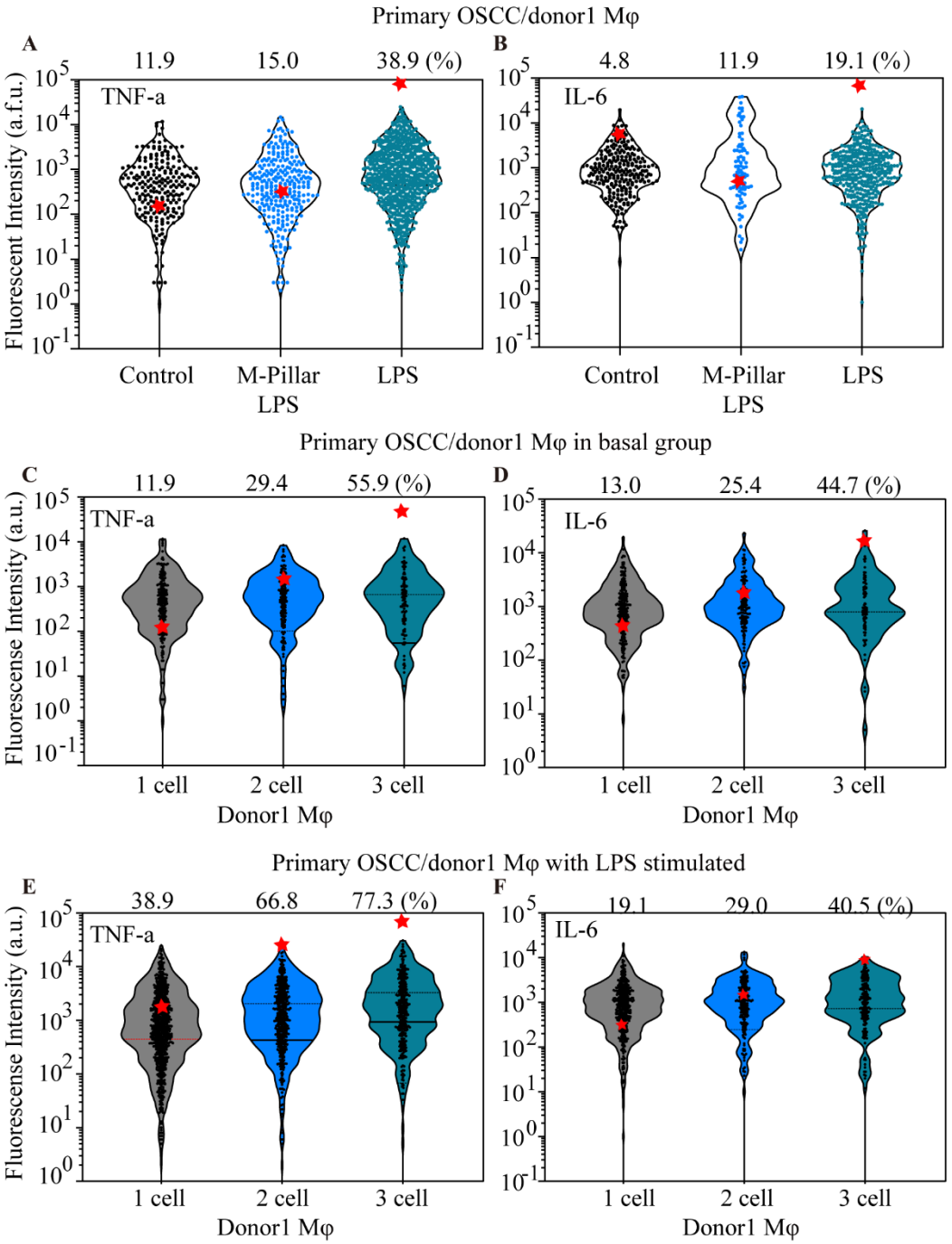

Figure S9. Secretion results in the tumor microenvironment with primary monocyte-derived macrophages from donor 1. A) TNF-a, and B) IL-6 secretion under different primary OSCC microenvironmental conditions: basal group (control), with LPS stimulated (LPS), and control experiment with only LPS stimulated macrophages (M-pillar LPS). C-F) The change of secretion frequencies with the increasing number of cells in each microchamber at the basal group (C, D), and stimulated with LPS (E, F).

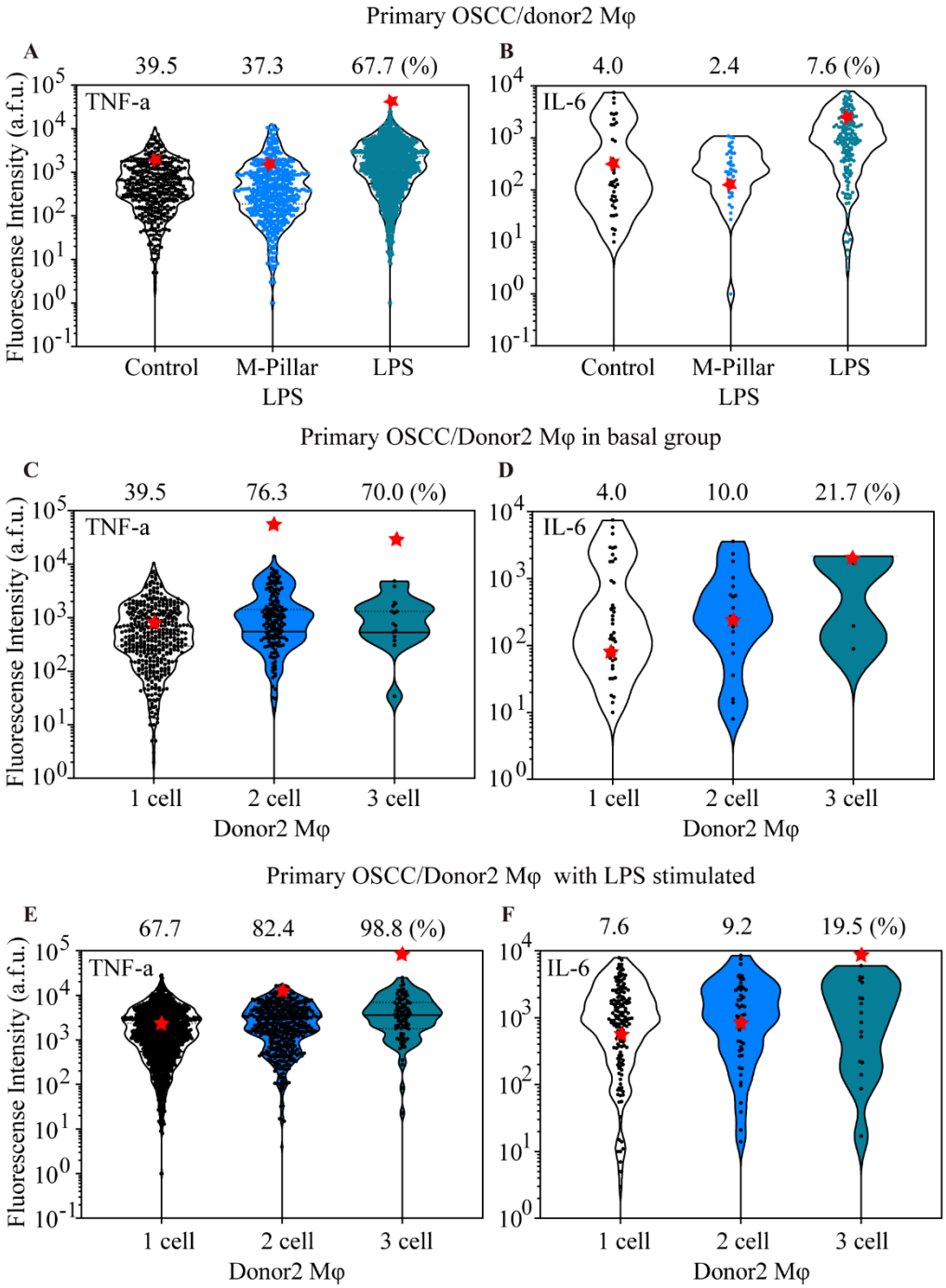

Figure S10. Secretion results in the tumor microenvironment with primary monocyte-derived macrophages from donor 2. A) TNF-a, and B) IL-6 secretion under different primary OSCC microenvironmental conditions: basal group (control), with LPS stimulated (LPS), and control experiment with only LPS stimulated macrophages (M-pillar LPS). C-F) The change of secretion frequencies with the increasing number of cells in each microchamber at the basal group (C, D), and stimulated with LPS (E, F).

Table S1. List of antibodies and staining reagents used.

Protein	Vendor	Product name	Catalog number
Human IL-6	Ebioscience	Human TNF alpha ELISA Ready-SET-Go!	88-7066-88
Human TNF-a	Ebioscience	Human TNF alpha ELISA Ready-SET-Go!	88-7346-88
Human MCP-1	Ebioscience	Human CCL2 ELISA Ready-SET-Go!	88-7399-22
MIP-1b	Ebioscience	Human CCL2 ELISA Ready-SET-Go!	88-703488
Human IL-8	Ebioscience	Human IL-8 ELISA Ready-SET-Go!	88-8086-88
Human IL-10	Ebioscience	Human IL-10 ELISA Ready-SET-Go!	88-7106-88
Human IL-6, FITC	Ebioscience	Anti-human IL-6, FITC	BMS130FI
Fluorescence Staining, 635	Ebioscience	Streptavidin APC	17-4317-82
Anti-CD68, PE	Ebioscience	Anti-human CD68, PE	12-0689-42
Anti-CD163, FITC	Biolegend	Anti-human CD163, FITC	333617
Anti-CD206, APC	Biolegend	Anti-human CD206, APC	321109

Table S2. Summary of the patient's medical records.

Gender	Age	Tumor type	Location	Differentiation	Metastasis
Male	55	OSCC	Mouth floor	High	Yes (cervical lymph node)

Table S3. Summary of donors' information.

Donor	Gender	Age	Height	Weight (kg)
1	Male	24	170 cm	69
2	Male	26	164 cm	72