

STAR+METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

C57BL/6 mice or BALB/c mice were purchased from Laboratory Animal Center of Southern Medical University. CD45.1 mice on a C57BL/6 background were purchased from GemPharmatech Co., Ltd. All mouse care and experiments were approved by the Animal Care and Use Committee of Southern Medical University (SMUL2022189), and all animals received humane care according to the standards outlined in the Guide for the Care and Use of Laboratory Animals.

Cell lines

Human CRC cell line (SW480, HT29, LoVo and RKO), human monocyte macrophage cell line (THP-1), Human embryonic kidney cell line 293T (HEK-293T) were cultured in RPMI1640 medium (Gibco) supplemented with 10% FBS (Gibco), 100 U penicillin/ml and 100 µg/ml streptomycin (Gibco) at 37°C in a humidified incubator with 5% CO₂. Mouse CRC cell lines (MC38, CT26), mouse melanoma cell line B16F10, mouse macrophage cell line (J774A.1, RAW264.7) and mouse connective tissue cell line (L929) and were cultured in DMEM medium (Gibco).

Human specimens

This study was approved by the Medical Ethics Committee of NanFang Hospital

of Southern Medical University (NFEC-2021-396). The experiments were undertaken with the understanding and written consent of each participant, which was in accordance with the Declaration of Helsinki. All human CRC tissue samples were collected from the Department of General Surgery, Nanfang Hospital, Southern Medical University. A tissue microarray (TMA), involving a total of 81 CRC patients who underwent colorectal resections from November 2013 to June 2014 in Nanfang Hospital, Southern Medical University (Guangzhou, China), was used to immunohistochemical (IHC) analysis and prognosis analysis. Twelve CRC samples prior to immunotherapy were used for IHC analysis. Ten CRC surgical specimens were used for immunofluorescence analysis. Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers.

METHOD DETAILS

Tumor growth and treatment

For the subcutaneous tumor xenograft models, CT26 colon cancer cells (5×10^5 cells or 1×10^6 per animal) or MC38 colon cancer cells (5×10^5 cells or 1×10^6 per animal) or B16-OVA melanoma cells (1×10^6 per animal) were subcutaneously (s.c.) injected into experimental mice to form solid tumors. For the orthotopic xenograft CRC mouse model, CT26 (5×10^5 cells) were injected into the wall of the cecum in BALB/c mice ($n = 5$ per group). After 19 days, all the mice were sacrificed. Colons were harvested to assess the tumor burden. For the AOM/DSS-induced murine colorectal carcinoma model, 6-week-old

female C57BL/6 mice were given a single injection of AOM (10 mg/kg in PBS; i.p.; Sigma-Aldrich, St. Louis, MO, USA). After 1 week of rest, mice were provided with 1.5% DSS (MP Biomedicals, Santa Ana, CA, USA) in their drinking water for 1 week, followed by DSS-free water for two weeks. The DSS treatment cycle was carried out three times in total, and mice were randomized into two groups and subjected to the therapeutic protocol. On week 13, the colon was removed and photographed. Tumors were counted and tumor size was measured using a caliper.

In some experiments, mice received intratumoral injection of MS4A4A siRNA (2.5 nmol/20g) to interfere with MS4A4A expression. Mice receiving intratumoral injection of control siRNA were included as controls. MS4A4A siRNA and control siRNA for in vivo experiments were purchased from Guangzhou Ruibo Biotechnology Co., LTD. In some experiments, mice received i.p. injection of clodronate liposomes (150 µl/animal, twice per week) or in combination with anti-CSF1R antibody (400 µg/animal, once a week) to deplete TAMs. In some experiments, mice received i.p. injection of anti-mouse CD8 antibodies (200 µg/animal, twice per week) to deplete CD8⁺ T cells. In some experiments, mice received i.p. injection of anti-mouse PD-1 antibodies (200 µg/animal, twice per week) to block PD-1, or received an i.p. injection of anti-mouse MS4A4A antibody (200 µg/animal, twice per week) to block MS4A4A; mice received i.p. injection of isotype antibodies was included as controls. In some experiments, mice received i.p. injection of anti-mouse

CD16/CD32 antibodies (200 µg/animal, twice per week) to block ADCC effect. In some experiments, mice were treated with radiation, 8Gy/day, every other day, for a total radiation dose of three times. Only tumor locations were exposed to radiation, and the body was shielded with a lead plate. During an experiment, tumor growth was monitored twice per week by measuring tumor size using a digital caliper; tumor volumes ($\text{length} \times (\text{width})^2 / 2$) were calculated. At the end of the experiment, solid tumors were collected and analyzed by qRT-PCR, IHC, flow cytometry and/or CyTOF.

In vivo macrophage adoptive transfer experiment

A total of 5×10^5 MC38 cells were injected subcutaneously into C57BL/6 mice on day 0 of the experiment. On day 1 of the experiment, GFP-labeled MS4A4A-overexpressing BMDMs (OE-MS4A4A-GFP) and control BMDMs (OE-NC-GFP) (5×10^5 cells per mouse) were injected intravenously into MC38 cell-challenged host C57BL/6 mice, respectively. On day 7 after tumor cell implantation, MC38 cell-challenged host C57BL/6 mice received the second macrophage adoptive transfer treatment administered via the same protocol as the first treatment. On day 7 after tumor cell implantation, MC38 cell-challenged host C57BL/6 mice received the second macrophage adoptive transfer treatment administered via the same protocol as the first treatment. Tumor dimensions were measured every two days beginning on day 6.

Macrophage suppressive function assay

BMDMs and splenocytes were harvested from C57BL/6 mice. MC38 cells were mixed with splenocytes and BMDMs in a 1:1:1 ratio. Cells were treated with 10 µg/ml MS4A4A antibody (3G7), in the presence of plate-bound anti-mouse CD3ε (5 µg/ml) and soluble anti-mouse CD28 (1 µg/ml) for 2 days. At the end of a culture, cells were collected for flow cytometry analysis.

Isolation of tumor-infiltrating cells

For single-cell suspension preparation, spleens and tumors of mice were harvested in DMEM medium containing 10% FBS. Tumor tissues were cut and collected in DMEM medium supplemented with 0.5 mg/ml collagenase IV (Sigma-Aldrich) and 0.1 mg/ml deoxyribonuclease type I (Sigma-Aldrich), incubated for 1 hour at 37°C in a shaker, and then mechanically dissociated on frosted glass slides. Harvested spleens were mechanically separated directly on frosted glass slides. Tumor or splenocyte suspensions were filtered through a 70 µm cell filter (Solarbio Life Science) to obtain single-cell suspensions. Lysate erythrocytes with 2 mL of RBC lysis buffer (Leagene) for 2 minutes at room temperature, then terminate the lysate with DMEM. Single-cell suspensions were counted and 1×10^6 cells were inoculated in 24-well plates for subsequent experiments. For cytokine analysis, cell activation cocktail with brefeldin A (BioLegend) was added to the cell culture medium to stimulate lymphocytes for 4-6 hours and then used for subsequent staining.

Flow cytometry

For surface staining, an appropriate amount of surface antigen antibody was added and incubated for 30 minutes according to the antibody instructions. For intracellular cytokine staining, cells were stained for cell surface antigens as described above. The cells were then incubated with fixation buffer (Biolegend) for 20 minutes at room temperature. The fixed cells were then resuspended in 1× intracellular staining perm wash buffer (Biolegend) and stained for intracellular cytokine. For intranuclear protein staining, the cells were resuspended with 1× FOXP3 Fix/Perm buffer (Biolegend), mixed, and incubated for 20 minutes at room temperature. Cells were resuspended with 1× FOXP3 Perm buffer (Biolegend) and incubated for 15 minutes at room temperature. The supernatant was discarded by centrifugation, and the cells were resuspended in 1× FOXP3 Perm buffer and stained for intranuclear protein. Uncompensated data were collected using a LSRFortessa cell counter with FACSDiva software (BD Biosciences). Compensation and analysis were performed using FlowJo v10.4.0 software. All antibodies used for flow cytometer analyses are listed in Key Resource Table.

Generation of anti-MS4A4A antibody

Anti-mouse MS4A4A (3G7) monoclonal antibody was customized at Aibixin Biotechnology Co., LTD. (Shanghai, China).

Preparation of conditioned medium (CM)

Preparation of conditioned media for L929 cells: L929 cells were cultured in medium dishes to 80% confluence, washed twice with PBS, added 10 ml of DMEM medium with 1% FBS, and continued to culture for 48 hours. The medium was collected, centrifuged, filtered to remove cell debris, and the culture supernatant was recovered. The CM was obtained by mixing the L929-primed medium with the regular medium (v/v = 1:4).

Preparation of tumor-conditioned media: colorectal cancer cells were cultured in a medium dish to 80% confluence, washed twice with PBS, added 10 ml of RPMI-1640 medium with 1% FBS, continued to culture for 48 hours and then collected the medium, centrifuged, filtered to remove cell debris. The CM was obtained by mixing the tumor-primed medium with the regular medium (v/v = 1:1).

Extraction of murine bone marrow-derived macrophages

6-8 weeks C57BL/6 mice were taken off the cervical vertebrae and executed, soaked in 75% ethanol. The skin of the legs was cut with scissors, and the muscles around each leg bone were thoroughly stripped. Trying not to destroy the integrity of the joint, and each leg bone was placed in 75% alcohol soak and then soak in PBS. Cut the ends of the leg bones and rinse out the cells in the bone marrow by aspirating DMEM medium with a syringe. Discard supernatant, add erythrocyte lysate and gently resuspend for about 2 minutes, followed by

DMEM medium with 3-5 times the volume of lysate to terminate lysis. 300g, centrifuge for 10 minutes. Resuspend cells with DMEM containing 20% L929 CM, count and spread in 6-well plates. The medium was changed once every 3 days. Generally, BMDM can be induced to mature after 6~7 days of culture.

Extraction of human PBMC derived macrophages cells

A total of 20ml peripheral blood was collected from healthy volunteers. 15 ml of lymphocyte separation solution was added to the central well of the lymphocyte separator SepMate-50 (Stemcell, Canada), and the whole blood was diluted 1:1 with PBS buffer and added to the upper layer of the separation solution, then the cells were collected by centrifugation at 2000 rpm for 20 minutes. Remove the supernatant and aspirate the middle white membrane. Add 5 times the volume of PBS buffer and centrifuge at 1500 rpm for 10 minutes. Add Monocyte Attachment Medium (PromoCell, Germany), resuspend and mix PBMCs, and spread in cell culture flasks. After 1.5 hours of incubation, the suspended cells were aspirated and macrophage basal medium (PromoCell, Germany) containing 50 ng/ml Recombinant Human M-CSF (Peprotech, USA) was added for 6 days, and the final walled cells were human PBMC-derived macrophages (PBMC-M).

Macrophage induction experiments in vitro

Induction of THP-1 differentiation into M1 macrophages and M2 macrophages:

THP-1 cells were differentiated into M0 macrophages by incubating with 50 ng/ml PMA for 48 h. For M1 polarization, M0 macrophages were stimulated with LPS (100 ng/ml) and IFN- γ (20 ng/ml) for 48 hours. For M2 polarization, M0 macrophages were stimulated with IL-4 (20 ng/ml) for 48 hours.

Induced differentiation of BMDM into M1 macrophages and M2 macrophages: For M1 polarization, BMDM were stimulated with LPS (100 ng/ml) and IFN- γ (20 ng/ml) for 48 hours. For M2 polarization, BMDM were stimulated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for 48 hours.

Induced differentiation of human PBMC-derived macrophages into M1 macrophages and M2 macrophages: For M1 polarization, human PBMC-derived macrophages were stimulated with LPS (100 ng/ml) and IFN- γ (20 ng/ml) for 48 hours. For M2 polarization, human PBMC-derived macrophages were stimulated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for 48 hours.

Induction of macrophages into TAMs: colorectal cancer cell conditioned medium was treated with the above macrophages for 24 hours, which resulted in colorectal cancer-associated macrophages (CRC-TAMs).

Quantitative real-time PCR

Total RNAs from cells or tissues were extracted with TRIzol reagent (TaKaRa) in accordance with the manufacturer's instructions. cDNAs were generated with PrimeScript RT-PCR Kit (TaKaRa). The expressions of mRNA were analyzed using SYBR Premix Ex Taq (TaKaRa) with a LightCycler 96 Detection System

(Roche) using GAPDH or β -actin for normalization. The primers used were detailed in Supplementary Table 1.

Western blot

Total protein was isolated using RIPA buffer (Amresco, USA) containing protease inhibitor cocktail. Protein extract was separated on SDS-PAGE gels followed by transfer to polyvinylidene fluoride membranes (Millipore, USA). The membranes were subsequently blocked in 5% defatted milk and incubated with primary antibody overnight at 4°C. Following incubation with the appropriate secondary antibody conjugated to horseradish peroxidase, the blots were visualised using the enhanced chemiluminescence (FDbio-pico ECL, China). All antibodies used for western blot are listed in Key Resource Table.

ELISA

ELISA was performed according to the manufacturer's instructions. In short, cells were inoculated into 96-well plates at the appropriate concentration and cultured for 24 hours. Then, cell culture medium were removed and replaced with an equal volume of serum-free DMEM. After 24 hours, the supernatant were collected and any floating cells were removed using a 0.45 μ m filter. The amount of secreted IL-10 and transforming growth factor (TGF)- β protein in the cell culture supernatant was measured using a mouse ELISA kit (Dakewe, China).

Immunohistochemistry staining (IHC)

IHC staining of paraffin-embedded human or mice tumor sections were performed according to standard protocols. Sections were deparaffinized, rehydrated, subjected to antigen retrieval, and blocked with 3% hydrogen dioxide and goat serum, followed by incubating in primary antibodies overnight at 4 °C. Primary antibodies used were described in Key Resource Table. Next day, the sections were put in room temperature for 30 minutes to rewarm, followed by secondary antibody incubation for 1 hours in room temperature and DAB staining was performed with IHC assay kit (Maixin, China). Counterstaining was carried with hematoxylin for 2 minutes. Images were taken with OLYMPUS DP22 microscope. Antibodies were shown in Key Resource Table.

The expression of indicated markers was assessed independently by two pathologists. MS4A4A density was calculated as density average of MS4A4A-positive cells observed in the different high-power fields from the same CRC sample (five fields for per CRC sample). Then, the average density of MS4A4A-positive cells was calculated for all CRC samples. When the number of MS4A4A positive cells was higher than this threshold, MS4A4A expression in these CRC samples was defined as high; otherwise, it was defined as low.

Multi-color IHC

Colorectal cancer surgery samples were provided by the Department of

General Surgery, Southern Hospital of Southern Medical University. Following a standardized application procedure, paraffin blocks were cut into 3-mm slices and glued to glass slides. The slides were baked in an oven at 65°C for 2 hours, dewaxed with xylene, and then hydrated with 100%, 95%, 85%, and 70% alcohol in that order. Multi-color IHC was performed using a four-color multiplex fluorescent immunohistochemical staining kit (Absin, Shanghai, China). Multi-color IHC was performed manually according to the manufacturer's protocol. Briefly, sections were subjected to microwave-induced antigen retrieval in sodium citrate buffer (PH = 6.0) and endogenous peroxidase blocking in 0.3% hydrogen peroxide in methanol. Then, sections were washed in TBST and blocked with goat serum for 30 minutes, incubated with primary antibody for 1 hour at room temperature, and slides were washed in TBST. Horseradish peroxidase-labeled goat anti-rabbit/mouse secondary antibody was used and was developed with fluorescent dye diluted by the signal amplification reagent provided with the kit. For multiple fluorescent staining, sections were processed starting from the antigen retrieval step to remove binding antibodies, and then they were incubated with another primary antibody. This was repeated until all antigens were stained. Finally, counterstaining was performed with DAPI and anti-fluorescence quenching blocker was added dropwise.

Stable cell line generation, plasmids or siRNA transfection

Lentiviral vectors was constructed by Vigene Biosciences, Shandong, China.

MS4A4A-overexpressed vectors (LV-MS4A4A) and control vectors (LV-NC) were transfected into THP-1 cells, J774A.1 cells and RAW264.7 cells. Lentiviral transfection procedures were performed according to provided protocols. Mouse Ms4a4a plasmid and mouse Ms4a4a siRNA was synthesized by GENECHM Biotech at Shanghai, China. Plasmid transfection and siRNA transfection were performed. Briefly, cells were cultured in six-well plates, and 3 µg of plasmid or 50 nM siRNA was mixed with Advanced DNA/RNA Transfection Reagent (Zeta Life, USA) and let stand at room temperature for 15 minutes. The mixtures were then added to the wells of six-well plates and incubated for 24 hours. Cells were incubated for 48 or 72 hours after transfection before testing for transgene expression or performing downstream experiments.

CCK8 and colony formation assay

For the cell proliferation assay, cells were cultured in 96-well plates (1000 cells per well) and cell proliferation was detected at 450 nm for 5 days using Cell Counting Kit 8 (DOJINDO Laboratories). For the colony formation assay, differently treated cells (500 cells per well) were cultured in 6-well plates. At the end of the experiment, the formed colonies were washed with phosphate buffer (PBS), fixed in methanol and stained with 0.1% crystalline violet. Colonies containing more than 50 cells per well were counted (in triplicate).

RNA-seq

RNA was extracted as above, and library construction and sequencing were performed at Guangzhou RiboBio Co. Ltd. with an Illumina HiSeq 2500 sequencer. Briefly, reads from the RNA-seq data were aligned to the Ensembl v76 (mm10) transcript annotations using bowtie2 (v2.4.1) and RSEM (v1.2.18). Tag counts were normalized for GC content using EDASeq (v2.0.0). Significantly differential transcript expression was determined using DESeq2.

Mass cytometry (CyTOF) analysis

Fresh mouse tumor samples were subjected to CyTOF analysis. Tumor samples were dissociated with the GentleMACS system (Miltenyi Biotec; Bergisch Gladbach, Germany) according to the manufacturer's instructions. Cells were then collected and stained with 41 antibodies, including CD45, CD3e, CD103, Fas, MHCII, CD44, Gr1, CD206, Ly6G, Ly6C, CX3CR1, CD127, ICOS, CD19, SiglecF, Ki67, CD11c, CD39, TIGIT, B220, F4/80, TCRb, iNOS, PDL1, CD25, CCR2, CD64, TCRgd, CD49b, CD27, CD62L, Tbet, CD69, PD-1, CD172a, Gata3, KLRG1, Tim3, CD4, CD8, and CD11b. Data were analyzed using the x-shift algorithm and separate t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction visualization. Antibody clones and suppliers are listed in Key Resource Table.

Statistical analysis

Results are presented as means \pm SEM unless stated otherwise. Survival

curves for different groups of mice were generated using the Kaplan-Meier method. A two-tailed unpaired Student t-test was used to compare the variables of two groups, and one-way or two-way ANOVA were performed for multi-group comparisons. Significant differences were reflected with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. All analyses were carried out with GraphPad Prism (version 8.0).