Supplemental material

Table S1 Clinicopathological characteristics of CRC patients

Factors	Cohort 1 (n=50)	CD155 low (n=93)	CD155 high (n=48)
Age	61.5; 21-85	56.9; (14-88)	61.2; (24-86)
Gender			
Male	31 (62.0%)	54 (58.1%)	28 (58.3%)
Female	19 (38.0%)	39 (41.9%)	20 (41.7%)
Tumor location			
Colon	41 (82.0%)	43 (46.2%)	30 (62.5%)
Rectum	9 (18.0%)	50 (53.8%)	18 (37.5%)
Tumor diameter			
<5 cm	30 (60.0%)	52 (55.9%)	20 (41.7%)
≥5 cm	20 (40.0%)	41 (44.1%)	28 (58.3%)
T stage			
T1-2	8 (16.0%)	7 (7.5%)	4 (8.3%)
T3-4	42 (84.0%)	86 (92.5%)	44 (91.7%)
N stage			
N0	35 (70.0%)	37 (39.7%)	16(33.3%)
N1-2	15 (30.0%)	56 (60.3%)	32 (66.7%)
M stage			

M0	47 (94.0%)	92 (98.9%)	45 (93.7%)
M1	3 (6.0%)	1 (1.1%)	3 (6.3%)
TNM stage			
I/II	34 (68.0%)	36 (38.7%)	14 (29.1%)
III/IV	16 (32.0%)	57 (61.3%)	34 (70.9%)
CEA			
<5	34 (68.0%)	66 (70.9%)	29 (60.4%)
≥5	16 (32.0%)	27 (29.1%)	19 (39.6%)
Differentiation			
Well	2 (4.0%)	4 (4.3%)	1 (2.1%)
Moderate	48 (96.0%)	74 (79.5%)	36 (75.0%)
Poor	0 (0.0%)	15 (16.2%)	11 (22.9%)
Relapse			
N	N/A	73 (78.4%)	27 (56.2%)
Y	N/A	20 (21.6%)	21 (43.8%)
Survival			
N	N/A	11 (11.8%)	17 (35.4%)
Y	N/A	82 (88.2%)	31 (64.6)

Table S2 PCR primers, shRNAs, and the primers for genotyping used in this study.

Gene name	Forward primer (5'-3')	Reverse primer (3'-5')
Hu CD155	CCGTCCAGGTCAAAGGTACAG	GGGGTCTTCATCCATTGGGG
Mu CD155	TGTTAAGCGCCAGGGCAATA	CAGACCCTAGGGCATTGGTG
Hu CD206	ACCTGCGACAGTAAACGAGG	TGTCTCCGCTTCATGCCATT
Hu CD86	TCTACCGTCAGTCCTGGCAT	AGCAGCATTCCCAAGGAACA
Hu GAPDH	GGATTTGGTCGTATTGGGCG	TCCCGTTCTCAGCCATGTAG
Mu GAPDH	GCATCTTCTTGTGCAGTGCC	TACGGCCAAATCCGTTCACA
Hu CD155 (sh)	CCAATCAACACAACTTTAATCTGCA	
Mu CD155 (sh)	CGTCCAGTATTCATCTGTGAA	
CD155-ko (p1)	TCGACCCTCCCAAGACG	
CD155-ko (p2)	GGGGGTGGGATGCAGATGTAAA	
CD155-ko (p3)	GCCCTGGCTGTCCTGTAACTCG	

Materials and methods in detail

Processing of human samples

Flow cytometry: The cells from human samples were then stained with fluorochrome-conjugated anti-CD45, CD11b, CD86, CD206, CD155, TIM-3, LAG-3, PD-1, CD3, CD4, CD8, and goat anti-mouse PE-labelled secondary antibodies for 30 min at 4°C. Intracellular cytokines were detected after 4 h of stimulation with Leukocyte Activation Cocktail (BD Pharmingen, USA). Then, cells were permeabilized using fixation and permeabilization solution (BD Cytofix/Cytoperm, USA) according to the manufacturer's instructions. Subsequently, the sample was incubated with antibody (fluorochrome-conjugated anti-IL-10, IL-12, and TNF-α) in Perm/Wash buffer for 30 min at 4°C. Finally, the samples were washed three times in PBS and analyzed with flow cytometry (FACSymphony, BD Bioscience, USA). Data were collected as FCS files. Each treatment condition included at least 10,000 cells for analysis. The abovementioned antibodies for flow cytometric analysis were purchased from BioLegend (San Diego, CA, USA).

Immunohistochemistry and immunofluorescence staining: Consecutive tissue sections (5-μm-thick) were obtained from formalin-fixed and paraffin-embedded tissues. Briefly, after deparaffinization and rehydration, antigen retrieval was performed by heat treatment using EDTA buffer (0.25 mM, pH 8) in a water bath at 98°C for 15 min. Then, 10% bovine serum albumin (BSA) was used to block nonspecific binding by incubation for 30 min. Then, the sections were stained with anti-CD155 (Abcam, USA), anti-CD68 (Abcam), and anti-TGF-β (Servicebio, China) antibodies. To assess the staining of each antibody, a standard setting was applied for all slides. Finally, the sections were observed by an optical microscope or confocal laser scanning microscope (CLSM) (Nikon, Tokyo, Japan), and 5 regions of interest

were randomly selected for each group.

Cell lines and cell culture

The human CRC cell line HCT116, mouse CRC cell lines CT26 and MC-38, human monocyte cell line THP-1, and mouse macrophage cell line RAW264.7 were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and all cells were tested and authenticated for their genotypes by DNA fingerprinting. The cell lines used in the experiments were passaged for fewer than 6 months after reconstitution. The THP-1 cells were differentiated into macrophages by incubation with 100 ng/ml 12-myristate 13-acetate (PMA, Sigma, USA) in RPMI 1640 medium for 24 h. Macrophages were further polarized to M1 macrophages by incubation with 100 ng/ml lipopolysaccharide (LPS, Sigma, USA) and 20 ng/ml IFN-γ (Pepro Tech Inc, Rocky Hill, NJ, USA), and M2 macrophages were obtained by incubation with 20 ng/ml IL-4 (Pepro Tech Inc) and 20 ng/ml IL-13 (Pepro Tech Inc). All experimental cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin at 37°C in 5% CO_2.

Knockdown of CD155 in $CD155^+$ macrophages

Prior evidence has documented that the human monocyte cell line THP-1 and mouse macrophage cell line RAW264.7 were extensively employed to simulate models of human and mouse TAMs in vitro.[1,2] FACS analysis was performed to analyze the expression pattern of CD155 in human PBMCs-derived, THP-1-derived, mouse bone marrow-derived, and RAW264.7-derived macrophages. CD155 knockdown in THP-1 and RAW264.7 cells was performed by using a set of distinct CD155 shRNAs designed by HANBIO (Shanghai, China). The sh-CD155 sequences were listed in Supplementary Table 2. These non-overlapping shRNAs were packed into the lentiviral vector and added to THP-1 and RAW264.7 cells for 24 h. The medium was

replaced with fresh RPMI 1640 medium, and continued to culture for 72 h. Puromycin was utilized to select resistant colonies. The expression of CD155 in the stable cells was tested by RT-qPCR, Western blot assay, and flow cytometry. Among the tested CD155 shRNAs, the shRNA with the highest CD155 knockdown efficiency was used for the following experiments and named sh-CD155. The negative control lentiviral shRNA was named NC-CD155.

Stable colony testing: mRNAs from NC-CD155 THP-1, sh-CD155 THP-1, NC-CD155 RAW264.7, and sh-CD155 RAW264.7 cells were extracted, and cDNA was generated. The CD155 primers used were synthesized by Ruibio Tech (Guangzhou, China), and the primers were listed in Supplementary Table 2. Samples were run in duplicate, and all qPCR results were normalized to the levels of GAPDH. The mRNA extracted from THP-1 and RAW264.7 cells were used as a positive control. Western blot analysis was conducted to assess the expression of CD155 in NC-CD155 THP-1, sh-CD155 THP-1, NC-CD155 RAW264.7, and sh-CD155 RAW264.7 cells. Total protein was extracted, and the protein concentration was detected by a BCA kit. After separation on 10% SDS-PAGE gel, proteins were transferred to polyvinylidene difluoride (PVDF) membranes and incubated with an anti-CD155 antibody (CST, USA) and subsequently a secondary antibody. Then, CD155 proteins were visualized using an ECL kit. Proteins extracted from THP1 and RAW264.7 cells were used as positive controls. For flow cytometry, cells were harvested and stained with anti-human CD155-PE (BioLegend) or anti-mouse CD155-PE (BioLegend). The expression of CD155 was determined using flow cytometry.

Macrophage differentiation: THP-1 cells were differentiated into macrophages by 24 h of incubation with 100 ng/ml PMA in RPMI 1640 medium. NC-CD155

macrophages and sh-CD155 macrophages were further polarized to M1 macrophages by incubation with LPS and IFN-γ, and M2 macrophages were obtained by incubation with IL-4 and IL-13. After 48 h, the harvested macrophages were stained with fluorochrome-conjugated anti-CD86, CD206, CD68, and F4/80 antibodies. Antibodies for flow cytometric analysis in this part were purchased from BioLegend. RT-qPCR was conducted to evaluate CD86 and CD206 mRNA expression in each group. The CD86 and CD206 primers were synthesized by Ruibio Tech, and the primers were listed in Supplementary Table 2.

Enzyme-linked immunosorbent assay (ELISA): NC-CD155 macrophages and sh-CD155 macrophages were cultured in RPMI 1640 medium. To measure the concentrations of IL-10, TNF-α, IL-12, and TGF-β, macrophages were cultured at a density of 10^6/ml. After being cultured for 48 h, the culture media were collected for ELISA analysis. The concentrations of IL-10, TNF-α, IL-12, and TGF-β were detected by ELISA kits (MEIMIAN, China) according to the manufacturer's instruction.

Macrophages-CRC cell coculture

Migration and invasion assays: Transwells with 8 μm pore size and Matrigel (both from Corning Inc, New York, USA) were utilized. CRC cells were serum-starved for 24 h to avoid possible serum effects. NC-CD155 macrophages or sh-CD155 macrophages were resuspended in a 10% FBS growth medium and seeded in the lower chamber, CRC cells were resuspended in an FBS-free medium and seeded on the top chamber with or without Matrigel (ratio 3:1). Chamber with Matrigel was used for the invasion assay and a chamber without Matrigel was used for the migration assay. After 24 h of incubation at 37°C in 5% CO2, the non-invaded and non-migrated CRC cells were removed with a cotton swab. Cells on the bottom of the membrane

were fixed with 4% formalin and stained with 0.1% crystal violet. The cells were counted in five randomized fields under an optical microscope (Nikon, Tokyo, Japan) at 100× magnification. The transwell invasion and migration assays were repeated three times with technical duplicates. CRC cell-monoculture was used as a negative control.

Apoptosis analysis: A 24-well culture plate with transwell chambers (0.4 μm pore size; Corning) was used. NC-CD155 macrophages or sh-CD155 macrophages were resuspended in RPMI 1640 media and seeded on the top of the chamber, and CRC cells were seeded on the lower chamber (ratio 3:1). After 24 h, CRC cells were washed with PBS and harvested, and then stained with Annexin-V/propidium iodide (KeyGEN BioTECH, China) for flow cytometry analysis. CRC cell-monoculture was used as a negative control.

Cell cycle analysis: A 24-well plate with transwell chambers (0.4 μm pore size; Corning) was used. NC-CD155 macrophages or sh-CD155 macrophages were resuspended in RPMI 1640 media and seeded on the top of the chamber, and CRC cells were seeded on the lower chamber (ratio 3:1). After 24 h, these CRC cells were washed with PBS, and then fixed in ice-cold 70% ethanol for 24 h. After centrifugation to remove ethanol, the cells were stained with RNase A and propidium iodide (PI) (KeyGEN BioTECH) for 30 min at room temperature. DNA contents were then assessed by flow cytometry. CRC cell-monoculture was used as a negative control.

Cell proliferation analysis: A 24-well plate with transwell chambers (0.4 μm pore size; Corning) was used. NC-CD155 macrophages or sh-CD155 macrophages were resuspended in RPMI 1640 media and seeded on the top of the chamber, and CFSE-labeled CRC cells were seeded on the lower chamber (ratio 3:1). After 24 h,

these CRC cells were washed with PBS, and then assessed by flow cytometry. CRC cell-monoculture was used as a negative control.

Molecular mechanism of migration and invasion: Western blot analysis was conducted to investigate the mechanism of $CD155^+$ TAMs-mediated migration and invasion of CRC cells. Galunisertib (HY-13226, MCE, 10 μM), an inhibitor of TGF-beta receptor I kinase was used to inhibit TGF-beta signaling [3]. Tofacitinib (HY-40354A, MCE, 2.5 μM), an inhibitor of Janus kinase, was used to inhibit JAK/STAT3 signaling.[4] CRC cells were pretreated with Tofacitinib or Galunisertib for 1 h. Total protein was extracted from CRC cells cocultured with NC-CD155 macrophages or sh-CD155 macrophages in the transwell culture system. After separation on 10% SDS-PAGE gel, proteins were transferred to PVDF membranes and incubated with anti-MMP2 antibody (Abcam, USA), anti-MMP9 antibody (Affinity, USA), anti-STAT3 antibody (CST), anti-p-STAT3 antibody (CST), and anti-GAPDH antibody (CST). Then, the membranes were incubated with the corresponding secondary antibodies, and the proteins were visualized using an ECL kit. Protein extracted from HCT116 or CT26-monoculture was used as a negative control.

Macrophage-$CD8^+$ T cell coculture

Isolation of $CD8^+$ T cells: Human PBMCs were isolated from healthy subjects via density gradient centrifugation. Human $CD8^+$ T cells were sorted and purified using microbeads coated with anti-CD8 (Miltenyi Biotech, Germany). For mouse $CD8^+$ T cells, normal BALB/c mice (5 weeks, male, bodyweight 20-22 g) were sacrificed, and spleens were collected to filtered through a 70 μm cell strainer to prepare a single-cell suspension. The use of mice for this experiment was approved by the Ethical Committee of Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China.

Similarly, mouse mononuclear cells were obtained using lymphocyte separation medium (DAKEWE, China), and mouse $CD8^+$ T cells were directly isolated using anti-CD8a microbeads (Miltenyi Biotech) according to the manufacturer's protocol. The isolated $CD8^+$ T cells were identified by incubating with antibodies against human CD8-BV510 (BioLegend, USA) or mouse CD8a-APC (BioLegend), and the purity was measured by flow cytometry analysis.

$CD8^+$ T cell function: The purified T-cell suspension was labeled with CFSE (5 μM, Invitrogen, USA). $CD8^+$ T cells were seeded in a 24-well plate precoated with 1.0 μg/ml anti-CD3 antibody (BioLegend, USA). NC-CD155 macrophages or sh-CD155 macrophages were resuspended in RPMI 1640 media and added to the $CD8^+$ T-cell culture system at a ratio of 1:1. Then, 2 μg/ml anti-CD28 antibody (BioLegend, USA) was added to the macrophage/$CD8^+$ T cell co-culture system. After 72 h, the cells were harvested and stained with fluorochrome-conjugated anti-CD8, IFN-γ, and GZMB antibodies. Cells were sorted for flow cytometry analysis.

CRC cells-primary macrophages coculture

Isolation of monocytes: Human PBMCs were isolated from healthy subjects via density gradient centrifugation. Human monocytes were sorted and purified using microbeads coated with anti-CD14 (Miltenyi Biotech). The isolated cells were cultured in RPMI 1640 medium containing 10% FBS supplemented with macrophage colony-stimulating factor (M-CSF, 50 ng/mL; PeproTech) at 37°C in 5% CO2. The medium was replaced every 2 days, monocytes were fully differentiated into macrophages after 7 days, and the macrophages in a stable growth state were collected for subsequent experiments. Mouse bone marrow cells were harvested from normal BALB/c mice (5 weeks, male, bodyweight 20-22 g). Bone marrow was flushed out into cold PBS and then passed through a 70-μm cell strainer to remove

cell clumps and other tissues. Then, the cells were incubated with RBC lysis solution to remove red blood cells. The harvested cells were cultured in RPMI 1640 medium containing 10% FBS and supplemented with mouse macrophage colony-stimulating factor (M-CSF, 50 ng/mL; Pepro Tech) for 7 days to obtain bone marrow-derived macrophages. The proportion of macrophages was detected using flow cytometry.

CD155$^+$ macrophage proliferation: The primary macrophages were seeded into the lower chamber (0.4 μm pore size; Corning) in a 24-well plate, and CRC cells were resuspended in RPMI 1640 media seeded on the top of the chamber (ratio 1:1). At the same time, M1 (100 ng/ml LPS and 20 ng/ml IFN-γ) and M2 (20 ng/ml IL-4 and 20 ng/ml IL-13) polarization were used as positive controls in this study. After 48 h, the harvested human macrophages were stained with anti-human CD11b-FITC, anti-human CD86-PerCP/Cyanine5.5, anti-human CD206-BV421, and anti-human CD155-PE, and mouse macrophages were stained with anti-mouse F4/80-APC, anti-mouse CD86-Briliant Violet 605, anti-mouse CD206-Briliant Violet 421, and anti-mouse CD155-PE. Antibodies for flow cytometric analysis in this part were purchased from BioLegend.

Cytokines detection by ELISA: CRC cells (HCT116 and CT26) and normal intestinal epithelial cells (NCM460 and IEC-6) were cultured in RPMI 1640 medium. To measure the concentrations of IL-4 and IL-13, cells were cultured at a density of 10^6/ml. After being cultured for 48 h, the culture media were collected for ELISA analysis. The concentrations of IL-4 and IL-13 were detected by ELISA kits (MEIMIAN, China) according to the manufacturer's instructions.

IL-4 neutralized by a monoclonal antibody: The activity of human IL-4 is neutralized by a mouse anti-human IL-4 monoclonal antibody (69005-1-Ig, Proteintech, USA), and the half neutralization dose (ND50) is typically 200-500ng/ml

according to the manufacturer's instruction (we chose 400ng/ml for the experiments). The mouse IL-4 is neutralized by monoclonal antibody (MAB404-100, R&D, USA), and the ND50 is typically 100-600ng/ml according to the manufacturer's instruction (we chose 500ng/ml for the experiments). The primary macrophages were seeded into the lower chamber (0.4 μm pore size; Corning) in a 24-well plate, and CRC cells were resuspended in RPMI 1640 media seeded on the top of the chamber (ratio 1:1). Primary macrophages were pretreated with these neutralizing antibodies for 2 h and then cocultured with CRC cells. After 48 h of incubation at 37°C in 5% CO2, the harvested primary macrophages were sorted for flow cytometry analysis.

In vivo study

Animal model: BALB/c mice (5 weeks, male, bodyweight 20-22 g) were purchased from Yancheng Biotechnology Co., Ltd (Guangzhou, China). CD155 knockout mice ($Cd155^{-/-}$) and wild-type C57BL/6J mice (C57, 5 weeks, male, bodyweight 20-21g) were purchased from Shanghai Model Organisms (Shanghai, China). All mice were maintained under specific pathogen-free conditions with free access to water and a chow diet in the animal facility. Animal experiments were performed according to the Institutional Animal Care and Use Committee of Sun Yat-sen University. This study was approved by the Ethical Committee of the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China. To establish the subcutaneous tumor model, each mouse was inoculated subcutaneously in the right front sub-axillary region with tumor cells in 0.1 ml of PBS (3×10^5 CT26 cells in BALB/c or MC-38 cells in C57). Tumor size and mouse weight were recorded every day, and tumor volume was calculated by the modified ellipsoidal formula as follows. Tumor volume: length × $width^2\times0.5$.

Bone marrow transplant (BMT): To identify whether CD155 expressed by

macrophages regulates antitumor immunity, BM chimeric mice were generated. C57 wild-type mice were irradiated twice (RS2000pro-225, 10 Gy), and BM cells from nonirradiated $Cd155^{-/-}$ mice (2×10^6) were injected i.v. into each irradiated mouse to generate BM chimeric mice. After 2 weeks of BMT, mouse blood genotypes were verified by agarose gel electrophoresis. Then, a subcutaneous tumor model was established in BM chimeric and wild-type mice by MC-38 cells.

Genotype analysis

Total DNA was extracted from PBMCs of $Cd155^{-/-}$ BMT C57 mice and wild-type C57 mice using the Blood Genomic DNA Extraction Kit (Accurate Biotechnology, China) according to the manufacturer's instructions. For the analysis of the genotypes, PCR was performed using a PCR mix (Dongsheng, China), and the specific primers were listed in Supplementary Table 2. PCR products were separated using 2% agarose gel electrophoresis.

Tumor growth analysis: After 1 week of CRC cell inoculation, the tumor volume reached approximately 50 mm^3. Then, the original macrophages in the BALB/c mice were depleted by clodronate liposomes (F70101C-A-10) injected intraperitoneally twice a week.[5] One day after clodronate liposome injection, BALB/c mice were injected with NC-CD155, or sh-CD155 macrophages via the tail vein (1×10^6 macrophages in 50 μl PBS, every 3 days for 7 times). The tumor volume and body weight were recorded every 3 days. When the average tumor size reached 2,000 mm^3, tumors were harvested for subsequent immunohistology and tumor immune microenvironment (TIME) analysis.

Immunohistochemical and immunofluorescence staining: At the end of the treatment, mice were sacrificed, and tumors were collected for formalin fixation to prepare paraffin-embedded sections. For immunohistochemical and

immunofluorescence analysis, sections were firstly deparaffinized and rehydrated and tissue sections were blocked with 10% BSA. Then, the sections were stained with anti-Ki67 (Servicebio), anti-CD31 (Servicebio), anti-TUNEL (Servicebio), anti-MMP2 (Abcam), anti-MMP9 (Affinity), and anti-TGF-β (Servicebio) antibodies. Finally, the sections were observed with an optical microscope or CLSM, and 5 regions of interest were randomly selected for each group.

TIME monitoring in a mouse model: To analyze changes in immune cells in TIME, the harvested tumors were collected, and single-cell suspension was prepared for flow cytometry analysis. For analysis of TAMs, single-cell suspensions from tumor tissues were stained with fluorescent-labeled anti-CD11b, CD86, CD206, CD155, and F4/80 antibodies (BioLegend). For T-cell analysis, single-cell suspensions from tumor tissues were stained with fluorescent-labeled anti-CD3, CD4, CD8, IFN-γ, and GZMB antibodies (BioLegend). Cells were sorted for flow cytometry analysis. MMPs and TGF-β from tumor tissues were determined by IHC, and the expression pattern of pSTAT3/STAT3 in tumor tissue was assessed by western blot assay.

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