

Supplementary Methods

Targeted lipidomics

Sphingosine (Cat. no. NS440602) and Sphinganine (Cat. no. 10007945) were obtained from Funakoshi Frontiers in Life Science (Tokyo, Japan). Phytosphingosine (Cat. no. P1765) and 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (Cat. no. D4213) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Sphingosine-1-phosphate (Cat. no. 860492P), Ceramide (d18:1/16:0) (Cat. no. 860516) and ceramide/sphingoid internal standard mixture II (Cat. no. LM 6005) were obtained from Avanti polar lipids (Alabama, USA). LC/MS grade methanol (Cat. no. 25185-76) and isopropanol (Cat. no. 32435-76) were obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan). Acetic acid (Cat. no. 533001), EDTA (ethylenediaminetetraacetic acid, Cat. no. E9884), and ammonium acetate (Cat. no. 533004), of LC/MS additive grade were obtained from Sigma-Aldrich (St. Louis, MO).

Single Cell RNA seq data analysis

Public sc-RNAseq data were analyzed from a previously published human colorectal cancer cohort comprising 62 patients. The dataset includes 371,223 quality-controlled cells profiled across 41,364 genes and encompasses malignant epithelial, immune, and stromal compartments. Immune populations include T, NK, and ILC subsets, B cells, plasma cells, mast cells, and myeloid lineages, while stromal populations include fibroblasts, endothelial cells, pericytes, and Schwann cells. Processed, normalized expression matrices provided by the original study were used to evaluate transcript abundance of S1P-related genes (SPHK1, SPHK2, S1PR1–5) across annotated cell populations. No additional re-clustering or batch correction was performed beyond the preprocessing reported in the source dataset. Cell types were grouped into major lineage classes for visualization and comparison.

Loupe browser analysis

Public single cell RNA-sequencing data a high-resolution image file (SVG format) from sc-RNAseq data was generated for each of the genes using Loupe Browser version 9 (Loupe Browser tutorial -Software -Single Cell Gene Expression -Official 10x Genomics Support), data for each gene was split between normal and tumor samples. Scale and attributes were “Log 2”. Loupe files can be downloaded from Human Colon Cancer Atlas (c295) – Single Cell Portal (broadinstitute.org). Raw data matrices for the Human Colon Cancer Atlas were downloaded from the Broad's Single Cell Portal. Raw counts were transformed log (TP10K+1) and merged with provided tSNE coordinates and ClusterTop annotations. The bar plot shows mean normalized expression $\pm$ SEM by cell type, with the Myeloid-versus-Stromal comparison evaluated using a two-sided Mann-Whitney U test and Benjamini-Hochberg correction.

Visium spatial transcriptomic analysis

Spatial transcriptomic profiling was performed using the 10x Genomics Visium HD platform at 8 μ m bin resolution on primary colorectal cancer (CRC) tissue and matched CRC liver metastasis specimens. For the primary CRC sample, 340,769 8 μ m bins were detected under tissue. The dataset achieved a mean of 1,319.4 reads per bin and 31.2 UMIs per bin, with 17,239 genes

detected. A total of 95.9% of reads mapped to the probe set. These metrics indicate robust probe capture efficiency and gene detection at high spatial resolution.

For the CRC liver metastasis sample (2G_HD), 680,598 8 μm bins were detected under tissue with a mean of 732.5 reads per bin and 19.6 UMIs per bin. A total of 17,731 genes were detected. Mapping efficiency was high, with 96.9% of reads mapped to the probe set, 95.7% mapped confidently, and 93.1% mapped confidently to the filtered probe set. Reads half-mapped and split-mapped to the probe set comprised 1.1% and 0.1%, respectively. Sequencing generated 498,554,703 reads with 87.8% valid barcodes and 100% valid UMIs. Sequencing saturation was 96.8%, and base quality metrics exceeded Q30 for >95% of barcode, probe read, and UMI bases. The fraction of bins under tissue was 96.9%, with 306,865 UMIs per mm^2 and 9,977,804 reads per mm^2 of tissue. An additional sequencing run yielded 449,623,649 reads with 96.9% sequencing saturation, 96–97% Q30 base quality, 91.0% reads mapped confidently to the filtered probe set, and consistent UMI and mapping performance. Across runs, mapping metrics remained above 90%, indicating high alignment confidence.

Spatial gene expression analysis was performed using Space Ranger (10x Genomics) with default alignment and probe-set parameters. Gene-level expression values were normalized per bin according to the Space Ranger pipeline. Differential expression between spatially annotated compartments was computed within Space Ranger outputs, and log₂ fold-change (log₂FC) values were derived directly from bin-level normalized counts using the Space Ranger statistical framework (Wilcoxon rank-sum testing with multiple testing correction). Significant log₂FC values reported in figures correspond to adjusted p-values generated by the pipeline. Bivariate Moran's I statistics were calculated to assess spatial co-localization patterns of gene expression across tissue compartments. Bivariate Moran's I statistics were calculated to quantify spatial co-localization between pairs of gene expression features across Visium HD spots. This metric assesses whether high expression of one gene in each spatial unit is associated with high (or low) expression of a second gene in neighboring spatial units, considering the spatial adjacency structure of the tissue grid. Spatial weights were defined based on spot adjacency (first-order neighborhood). The bivariate Moran's I coefficient ranges from -1 to +1, where positive values indicate spatial clustering (co-enrichment in neighboring regions), negative values indicate spatial segregation, and values near zero indicate no spatial association. Statistical significance was determined using permutation testing with random label reshuffling across spatial coordinates.

Western blot analysis

Whole cell protein lysates were obtained from adherent cells by adding RIPA buffer (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM EDTA, 2% NP-40, 2% sodium deoxycholate, and 0.2% SDS) supplemented with protease inhibitors. Protein concentrations were quantified by the Bradford assay (Bio-Rad). Equal amounts of protein were resolved on SDS-PAGE using precast gradient mini gels (Bio-Rad, Cat. no. 456-1085) in the presence of 4 $\times$ Laemmli loading buffer and subsequently transferred onto nitrocellulose membranes (Amersham, Cat. no. 10600002) via wet transfer as previously described. Membranes were blocked with blocking buffer (Rockland, Cat. no. MB-070) and incubated overnight at 4 $^{\circ}\text{C}$ with primary antibodies against SPHK1 (Invitrogen, Cat. no. PA5-22994) and Actin (Cell Signaling Technology, Cat. no. 3700S), both diluted in

blocking buffer. After washing, membranes were incubated for 1 hour at room temperature with fluorescent secondary antibodies (IRDye® 800CW Goat anti-Rabbit IgG, Cat. no. 926-32211, and IRDye® 680RD Goat anti-Mouse IgG, Cat. no. 926-68170; LI-COR), diluted in blocking buffer. Signals were visualized using the LI-COR Odyssey® CLx Imaging System, and band intensities were quantified with Image Studio 5.2. Protein expression levels were normalized to Actin as a loading control.

Immunofluorescence analysis

Freshly isolated colon tumors were fixed in 10% formalin for 48 h and then transferred to 70% alcohol. All further processing were carried out at Moffitt Histology Core. Briefly, samples were embedded in paraffin, and 5-micron sections were cut. Deparaffinization of tumor sections was followed by rehydration by graded ethanol steps and washes in 1x phosphate buffered saline (PBS). The sections were subjected to heat induced antigen retrieval (95°C for 20 min) using 10 mM citrate buffer, pH 6.0 and cooled to room temperature for 20 min. After PBS washes, the tissue were blocked in 10% normal goat serum (Jackson Immuno Research labs, Cat. No. NC9660079) in 1x PBS at room temperature for 1 hour. This was followed by incubation of sections with primary antibodies at the required dilutions at 4°C overnight. The primary antibodies used in this study are as follows. Rabbit SPHK1 polyclonal (1:100, 10670-1-AP, Proteintech), Mouse cytokeratin pan antibody cocktail (1:200, PIMA513320, Thermofisher scientific), Rabbit SIPR3 (1:50, ASR-013 Alomone labs), Mouse monoclonal CD163 (1:100, NB110-59993555, Novus Biologicals). Following washes in 1X PBS, the sections were incubated with appropriate fluorophore conjugated secondary antibodies at 1: 200 dilution. We used goat anti-rabbit 594, A-11012, Thermofisher scientific; goat anti-mouse 488, A-11001, Thermofisher scientific at room temperature for 1hour in dark. Following PBS washes, nuclei were stained with DAPI (1mg/ml, 62248, Thermofisher scientific) for 20 minutes and washed in PBS. The coverslip was applied to the slides using the Slowfade diamond antifade mountant (S36963, Thermofisher scientific) and sealed with nail polish. The images were acquired using confocal microscope (Agilent C10) and processed using Adobe photoshop version 11.

Real-time Cytotoxicity Assay

To analyze changes in the functional capacity of T cells cultured with treated macrophages, or their conditioned media, we performed cytotoxicity assays against adherent A549 target cells (ATCC CAT#CCL-185, RRID: CVCL_A549) as we have done previously ([1]). Cytotoxicity assay measurement was selected in the xCelligence Real-Time Cell Analysis (RTCA) instrument (ACEA Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. A549 target cells were added to each of a 96-well E-Plate (ACEA Biosciences, San Diego, CA, USA) at a concentration of 10^4 cells/well. Target cells were allowed to settle, and measurements of target cell growth (impedance) were continuously collected at 37°C, 5% CO₂. After 48 hours media was removed from the wells and fresh complete media with macrophages at 10^4 cells/well or conditioned media as described. Primary isolated T cells were also added onto target cells at 5×10^4 cells/well cells per well at an E:T ratio of 5:1. Data was analyzed for cytotoxicity against target cells only and normalized after target cell addition. Data for each treatment was done in triplicate wells of three separate donor cells. Data was exported to excel and analyzed as described in the statistics section.

Flow Cytometry

All experiments started with viability staining of cells collected from our experiments after washing twice with 1X PBS (Cat# 14190-250, Invitrogen, Carlsbad, California) and incubating with Live/Dead fluorescent reactive dyes (see specific sections below) according to the manufacturer's protocol in the absence of protein to avoid non-specific staining. After washing to remove the Live/Dead reagent, non-specific binding was blocked by incubating cells in flow cytometry staining buffer containing, 1X PBS supplemented with 0.5% bovine serum albumin (BSA, cat# 0332-500G, VWR life science, Radnor, Pennsylvania), 0.1% human male AB serum (cat# 515-H1, Access Cell Culture, Vista, California) and 0.1% mouse serum (cat# 31881, ThermoFisher Scientific, Waltham, Massachusetts). To block Fc receptors, we used the TrueStain FcX block (cat# 422302, Biolegend, San Diego, California) at room temperature for 15 min. Cells were subsequently stained with specific conjugated antibodies, diluted in the same buffer, for 1 hr. at room temperature. Following washing, cells were fixed with BD Cytofix/Cytoperm buffer (BD Biosciences, Franklin Lakes, NJ, Cat No. BDB554722) and washed with Perm/Wash buffer (BD Biosciences, Franklin Lakes, NJ, Cat No. BDB554723) for experiments requiring intracellular staining analysis. Sample acquisition was carried out using a BD LSRII flow cytometer located at Moffitt's Flow Cytometry Core equipped with the FACSDiva software (BD Biosciences, Franklin Lakes, NJ). Data was analyzed using FlowJo software (FlowJo, LLC., Ashland, Oregon, (FlowJo, RRID:SCR_008520). All panel used were designed in Fluorofinder for the filter configuration of the equipment used at the Flow Cytometry Core. The following antibodies were used in this study for flow cytometry:

CD107a and IFN γ activation

To assess the functional activation of T cells under different conditions isolated T cells were cultured in the conditions described for each experiment and at collection the cells were co-incubated with Golgi plug (BD biosciences Cat. No. 555029), Golgi stop (BD biosciences Cat. No. 554724) and CD107a APC (Clone: H4A3, BD Biosciences Cat. No. 641581, AB_1645722) at which point tumor target cells A549 were added and co-cultured for 4 hours prior to surface staining with anti-human CD4 Brilliant Ultraviolet 395 (Clone: SK3, BD Biosciences, Cat. No. 563550, RRID: AB_2738273), anti-human CD8 PerCP-Cy5.5 (Clone: SK1, BD Biosciences, Cat. No. 341051, RRID: AB_400209). After fixation and permeabilization with BD Cytofix/Cytoperm, intracellular staining was performed with anti-IFN γ FITC (Clone: 25723.11, BD Biosciences, Cat. No. 340449, RRID: AB_400425). For viability Live/Dead Fix Near IR (775) (Thermo Fisher Scientific, Cat. No. L34976) was used prior to surface staining in serum free 1X PBS.

Migration assay

To assess changes in the migratory capacity of T cells with macrophages or conditioned media. T cells were plated at the top of a trans well and the bottom contained either differentiated macrophages or conditioned media as specified for each experiment shown. After culture cells at the bottom were collected and measured for the migration of different T cell population using the following antibodies: anti-human CD4 Brilliant Ultraviolet 395 (Clone: SK3, BD Biosciences, Cat. No. 563550, RRID: AB_2738273), anti-human CD8 PerCP-Cy5.5 (Clone: SK1, BD Biosciences, Cat. No. 341051, RRID: AB_400209). anti-human CD3 eFluor 450 (Clone: OKT3, Thermo Fisher Scientific, Cat. No. 48-0037-42, RRID: AB_1272055), and anti-human CD11b PE (Clone: ICRF44, BD Biosciences, Cat. No. 555388, RRID: AB_395789). For assessing the absolute number of migrated cells per μ L CountBright™ Absolute Counting Beads for flow cytometry (Thermo Fisher Scientific, Cat. No. C36950) was added to each tube prior to analysis in the cytometer following the manufacturer instructions.

Proliferation

For analysis on changes on the proliferation activity of T cells in cultured with either differentiated macrophages or conditioned media, prior to culture the T cells were pre-stained in 1X PBS for 30 minutes in the dark with CellTracker Blue (Thermo Fisher Scientific, Cat. No. C2110) following the manufacturer instructions. After washing twice with 1X PBS cells were placed in their respective experiments throughout the duration prior to collection and surface staining. At collection cells were washed of serum with 1X PBS and stained with the viability dye Live/Dead Fix Near IR (775, Thermo Fisher Scientific, Cat. No. L34976). After incubation, blocking and washing, cells were surface stained with the antibodies: anti-human CD3 eFluor 450 (Clone: OKT3, Thermo Fisher Scientific, Cat. No. 48-0037-42, RRID: AB_1272055), anti-human CD4 Brilliant Ultraviolet 395 (Clone: SK3, BD Biosciences, Cat. No. 563550, RRID: AB_2738273), anti-human CD8 APC (Clone: RPA-T8, BD Biosciences, Cat. No. 555369, RRID: AB_398595) and anti-human CD11b PE (Clone: ICRF44, BD Biosciences, Cat. No. 555388, RRID: AB_395789).

T cell phenotype

After co-culture with differentiated macrophages or with the macrophage conditioned media cells were collected and stained with Live/Dead Fix Aqua (Thermo Fisher Scientific, Cat. No. L34966) for viability, followed by blocking and surface staining with the antibodies: anti-human CD8 Brilliant Ultraviolet 496 (Clone: RPA-T8, BD Biosciences Cat. No. 564804, RRID: AB_2744460), anti-human CD45 Brilliant Ultraviolet 805 (Clone: HI30, BD Biosciences catalog number 612891, RRID: AB_2870179), CD127 Brilliant Violet 605 (BD Biosciences catalog number 562662, RRID: AB_2737706), CD4 PerCP-Cy5.5 (Clone: SK3, BD Biosciences, Cat. No. 341654, RRID: AB_400452), anti-human CD25 Brilliant Violet 421 (Clone: M-A251, BD Biosciences, Cat. No. 562442, RRID: AB_11154578), anti-human CD127 Brilliant Violet 650 (Clone: A019D5, BioLegend, Cat. No. 351326, RRID: AB_2562095), anti-human CD3 Brilliant Ultraviolet 737 (Clone: UCHT1, BD Biosciences, Cat. No. 612750, RRID: AB_2870081), anti-human PD1 APC (Thermo Fisher Scientific Cat# 17-2799-41, RRID: AB_10598512).

Table #2 Taqman assay primers

Gene	Assay ID
<i>SPHK1</i>	Hs00184211_m1
<i>SPHK2</i>	Hs01016543_g1
<i>S1PR1</i>	Hs05021992_s1
<i>S1PR3</i>	Hs00245464_s1
<i>S1PR4</i>	Hs02330084_s1
<i>S1PR5</i>	Hs00928195_s1
<i>ACTIN</i>	Hs01060665_g1
<i>CD163</i>	Hs00174705-m1
<i>IL10</i>	Hs00961622_m1

<i>MRC1</i>	Hs07288635_g1
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References

1. Trinh T, Adams WA, Calescibetta A, Tu N, Dalton R, So T, Wei M, Ward G, Kostenko E, Christiansen S, et al: **CX3CR1 deficiency-induced TIL tumor restriction as a novel addition for CAR-T design in solid malignancies.** *iScience* 2023, **26**:106443.