SUPPLEMENTARY MATERIALS:

Fig. S1.

Correlation of the highest and lowest quartiles of IL-10 expression with disease-specific survival (DSS) at 5 years in the TCGA Pan-Cancer of primary colon adenocarcinoma (COAD) dataset demonstrating improved DSS for colon cancer with low *IL10* expression ($P<0.05$), but no change in DSS for colon cancer for high vs low *PDCD1* and *HAVCR1*expression ($P>0.05$).

Fig. S2.

S2A – IL-10 cytokine was measured in TSC supernatant from n=5 tumors treated with control or αIL-10, with a marked reduction in the IL-10 detectable within the system.

S2B – Co-staining of IHC by EpCAM marking for tumor cells (red) and cleaved-Caspase-3 (CC3) for apoptotic cells (brown) indicates that the increased CC3 expression is occurring on tumor cells. $EpCAM^+$ $CC3^+$ cells are denoted with black arrows. Representative of n = 3 patient tumors.

S2C –Staining of consecutive slides of CD163 and HLA-DR. Cells positive in the same position on the consecutive slides are denoted by black arrows. Representative of n = 3 patient tumors.

S2D - Multiplex IHC was performed for T cells (CD3, green), PD-1 (red), and nuclei (DAPI, blue). Quantification of proportion of $PD\text{-}1^+$ $CD3^+$ cells are also shown (n = 6 patient tumors). Granzyme B^+ by tissue area similarly stained by IHC and quantified. Scale bar = 50 µm. Data points represent each human tumor sample. Student's t test.

Fig. S3.

Two separate patient tumors were collected on sequential days 1, 2, and 3 of treatment with IL-10 blocking antibody or control antibody. Single cell RNA sequencing was performed on all samples. Violin plots of gene expression of cytolytic and activation gene signatures in the in $CD8^+$ and $CD4^+$ T cell subsets as well as TAM subset. The activation gene signature consists of *CD69*, *FASLG*, *CD27*, *CD28*, *TNFRSF9*, *TNFRSF4*, *LAMP1*, *EOMES*, *TBX21*(A-B). The exhausted gene signature consists of *PDCD1, CTLA4, HAVCR2, TIGIT*, and *LAG3* (C). Last, violin plot of the MHC-II presentation genes in the Reactome pathway in the macrophage cell cluster (D).

Fig. S4.

Results of low dose (10^5 rather than 10^6) CAR-T cell showing similarly increased CAR-T cell migration and CAR-T cell mediated carcinoma cell apoptosis which did not meet statistical significance.

Fig. S5.

Representative flow cytometry plot and quantification of cells gated for $CFSE^+$ T cells which are also positive for CD25 and/or CD69 from experiments depicted in Fig. 6C.

Sullivan KM, *et al. Gut* 2022;0:1–13. doi: 10.1136/gutjnl-2021-325808

Supplemental Materials and Methods

Tumor slice culture

Slice culture of freshly resected human CRLM tumors was performed as previously described [35 36 40]. Briefly, sterile cores from tumor specimens were obtained using a 6 mm punch biopsy in the operating room immediately following resection, under the review of the on-call surgical pathologist to ensure the retention of adequate tissue for diagnosis and margin assessment. The cores were placed in Belzer UW Cold Storage solution on ice. Using a vibratome (Leica Biosystems), 250 µm slices were cut sequentially and placed on a permeable PTFE membrane with 0.4 µm pores (Millicell, MilliporeSigma, Burlington, MA), placed in a 24-well dish containing RPMI media with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin but without any additional immune-activating factors or cytokines. Slice cultures were incubated overnight at 37°C and then treated with 10% FBS RPMI media containing either 20 µg/mL of IgG isotype control (#553447, BD Biosciences), 20 µg/mL of αPD-11 (#562138, Biolegend), or 20 µg/mL of αIL-10 (#501407, Biolegend) For an individual tumor sample, slices were distributed evenly and randomly from throughout the tumor with 3 slices per treatment group to account for confounding from intra-tumor heterogeneity. Slices were only excluded for technical failure of slicing and no treated slices were excluded. Treatment samples were not blinded when analyzing results. The dose of αIL-10 was chosen based upon a dose titration experiment using 5, 10, and 20 µg/ml concentrations of antibody, showing a maximal effect at 20 µg/ml. Additional experiments used 20 µg/mL of anti-MHC class I (purified mouse anti-human HLA-ABC #555551, BD Biosciences) and/or 20 µg/mL of anti-MHC class II (purified mouse anti-human HLA-DR, DP, DQ #555557, BD Biosciences). If the slices were to be maintained in culture for more than 3 days, half the volume of media was changed with fresh treatment after 72 hours.

In situ hybridization (ISH) and multicolor immunohistochemistry (mIHC)

For ISH, FFPE tissues were sectioned at 4 µm onto positively-charged slides and baked for 1 hour at 60°C. The slides were then dewaxed and stained on a Leica BOND Rx Stainer (Leica, Buffalo Grove, IL) using Leica Bond reagents for dewaxing (Dewax Solution), antigen retrieval with Epitope Retrieval Solution 2 for 15 minutes at 95°C, cell permeabilization with 0.5% Triton-X in PBS for 30 minutes at RT and rinsing after each step (Bond Wash Solution). All steps after epitope retrieval were performed at RT. The slides were then blocked with hydrogen peroxide for 10 minutes and incubated with the ACD target probe, IFN-g (#550478-C3, Advanced Cell Diagnostics) or IL10Ra (#498058, Advanced Cell Diagnostics) and IL10 (#602058-C2, Advanced Cell Diagnostics), at 42°C for 120 minutes. After the probe incubation, the staining proceeded with the ACD RNAscope LS Multiplex Fluorescent Reagent Kit (#322800) for the amplification and detection steps, followed by Opal fluor (Opal 650 for IFN-g, Opal 570/650 for IL10RA/IL10) at 1:750 for 10 minutes. Slides were removed from the Stainer and stained with DAPI for 5 minutes, rinsed for 5 minutes, and a coverslip was mounted with Prolong Gold Antifade reagent (Invitrogen/Life Technologies, Grand Island, NY). Slides were imaged with Leica SP8 confocal microscope.

For the mIHC staining, the coverslip of slides was removed in deionized water and stained on a Leica BOND Rx using Leica Bond reagents for dewaxing (Dewax Solution), antibody stripping (Epitope Retrieval Solution 2), and rinsing after each step (Bond Wash Solution). Antibody stripping steps were performed at 100°C with all other steps at ambient temperature. Endogenous peroxidase was blocked with 3% H_2O_2 for 5 minutes followed by protein blocking with TCT buffer (0.05M Tris, 0.15M NaCl, 0.25% Casein, 0.1% Tween 20, 0.05% ProClin300 pH 7.6) for 10 minutes. The first

primary antibody (position 1) was applied for 60 minutes followed by the secondary antibody application for 10 minutes and the application of the tertiary TSA-amplification reagent (PerkinElmer OPAL fluor) for 10 minutes. The primary antibodies used were EpCAM (Opal 690, 32402, Biolegend) in position 1, HLA-DR (Opal 620, M0746, Dako) in position 2, CD3 (Opal 570, RM9107, ThermoFisher) in position 3, and CD163/68 (Opal 540, BSB3276, BioSB and M0876, Dako) in position 4 followed by the Powervision Mouse-HRP secondary antibody (Leica). A high stringency wash was performed after the secondary and tertiary applications using high-salt TBST solution (0.05M Tris, 0.3M NaCl, and 0.1% Tween-20, pH 7.2-7.6). Polymer HRP was used for all secondary applications, as specified in the table. The primary and secondary antibodies were stripped with retrieval solution for 20 minutes before repeating the process with the second primary antibody (position 2) starting with a new application of 3% H_2O_2. The process was repeated until all positions were completed; however, there was no stripping step after the final position. Slides were removed from the stainer and stained with DAPI for 5 minutes, rinsed for 5 minutes, and coverslip mounted with Prolong Gold Antifade reagent (Invitrogen/Life Technologies, Grand Island, NY). Slides were imaged with Leica SP8 confocal microscope.

For combination of mIHC and ISH mIHC staining was done either before or after ISH. Slides were imaged after each step. ISH labeling was bleached when mIHC was performed subsequently. To combine the signals from ISH and mIHC, images were overlaid, and areas of perfect nuclear overlap were analyzed. Images were analyzed using Imaris (Bitplane). Parameters for each segmented cell including area, sphericity, mean intensity values were exported as comma separated value (CSV) files. Mean intensity values were normalized via z-score normalization. CSV files were analyzed via Flowjo (Beckton Dickinson).

Reverse phase protein array (RPPA)

Protein microarrays were printed and processed as described previously [41 42]. Briefly, tumor slices were lysed in 2% SDS lysis buffers as described previously[41 42]. Tumor slice lysates were printed onto 16-pad nitrocellulose coated slides (Grace Biolabs, OR, US) using Aushon 2470 microarrayer (Aushon BioSystems, MA, US). Each sample was printed in duplicate, and slides were stored at – 20°C until processing. Slides were then washed 2-3 times with PBS for 5 min each and blocked with Odyssey Blocking Buffer (OBB, Licor, NE, USA) for one hour at room temperature. After blocking, arrays were incubated with primary antibodies in OBB at 4°C overnight. The next day, arrays were washed thrice with PBS and incubated with IRDye labeled secondary antibodies in OBB for 1 hour at room temperature. Following incubation, slides were scanned using Licor Odyssey CLX Scanner (LiCOR). Total signal intensity from each spot was quantified using Array-Pro analyzer software package (Media Cybernetics, MD, USA). The measurement of a specific protein from an individual sample was normalized to total beta-actin (Sigma, Cat #A1978).

Immunohistochemistry (IHC)

At the desired times following treatment, slices were removed from culture and fixed in 10% formalin. Tissue was then paraffin embedded and 4 µm thick tissue slices were mounted on slides for storage. Slides were de-paraffinized in xylene and rehydrated through graded ethanol followed by heat mediated antigen retrieval using 10 mM Sodium Citrate Buffer (pH 6.0) and blocked with 3% hydrogen peroxide for 5 minutes and Background Buster (Innovex Biosciences) for 30 minutes. IHC was performed via incubations with one primary antibody at 4°C overnight followed by host-matched secondary polymer reagent and color substrate. Primary antibodies used were as follows: CD8 (M7103, Dako), HLA-DR

(M0746, Dako), CD163 (ab189915, Abcam), and cleaved-Caspase-3 (#9661, Cell Signal Technologies). Secondary reagents were ImmPress Rabbit HRP and Mouse HRP (Vector Laboratories). Color development was performed using DAB Quanto (Brown HRP; Fisher Scientific). Slides were counterstained with Harris hematoxylin (Sigma) as appropriate, dehydrated, and mounted. The transversely cut slides were imaged over multiple 20x hpf to visualize the entire thickness of the slice.

For dual-color IHC staining, sections were first stained as above with a primary antibody against cleaved-Caspase-3 (#9661, Cell Signal Technologies) and host-match secondary antibody Immpress Rabbit HRP (Vector Laboratories). Color development was performed using DAB Quanto (Brown HRP; Fisher Scientific). Slides were washed with TBST and PBS washing buffers, re-blocked with Background Buster for 15 minutes, and re-incubated with EpCAM (ab124825, Abcam) as the primary antibody. Immpress alkaline phosphatase (AP) anti-rabbit polymer (Vector Laboratories) was then used as secondary antibody and color development was performed with Warp Red substrate (WR 806CHC, Biocare). Slides were washed with TBST and PBS and counterstained with Harris hematoxylin (Sigma) and directly mounted with Aqua-mount (13800, Lerner Laboratories).

Stained tissue sections were digitally scanned and uploaded to NanoZoomer Digital Pathology (Hamamatsu) image viewing software. Images we exported and positive DAB cells and hematoxylin-stained nuclei were visually counted using Fiji ImageJ software. Each data point represents one case consisting of multiple (at least 2) slices and a minimum of 2 hpf within a single slice to account for both intra- and inter-tumoral heterogeneity.

Mice, liver metastasis (LM) in vivo model and myeloid cell isolation

C57BL/6 male mice (6–8-week-old) obtained from Jackson Laboratories were bred and maintained under pathogen free conditions at Roger Williams Medical Center (RWMC) animal facility. All surgical procedures were approved by RWMC Institutional Animal Care and Use Committee (IACUC). To generate LM, mice were anesthetized and injected with $2.5x10^6$ MC38CEA (generous gift from Dr. Jeffrey Schlom) via spleen followed by splenectomy to confine metastases to the liver. Liver non-parenchymal cells were isolated from LM, four days post-tumor generation, as previously described[15]. Immunomagnetic beads against CD11b were used to purify bulk hepatic MDSCs (Miltenyi Biotech). Typical purity of beaded $CD11b^+$ cells were 80–90% from mice with LM.

CAR-T generation, proliferation assay and cytotoxicity assay

CAR-T cells were generated from mouse splenocytes as described previously[43] and MC38CEA cells were used as target tumor cells. Isolated myeloid cells were isolated from LM tumor bearing mice livers. CAR-T cells were carboxyfluorescein diacetate succinimidyl ester (CFSE, Life Technologies) labeled as per company's protocol. MC38CEA cells were plated at $2x10^4$ cells/well in 96-well cell culture plate (BD Bioscience) with myeloid cells + CAR-T cells in a 1:1:1 ratio. Cells were treated with 100ng/ml or 200 ng/ml and 400 ng/ml of IL-10 antibody (JES5-2A5, Biolegend). After 24 hours, cells were removed and analyzed for CFSE using flow cytometry. Supernatant was collected and analyzed for LDH as per manufacturer's protocol (Promega). No exclusion of data points was necessary and treatment groups were not able to be blinded during analysis. N = 3 biological replicates and n = 4 technical replicates were performed.

Flow cytometry

Antibodies specific for the following markers were used: CD3 (17A2, BD Bioscience), IL-10 (JES5-16E3, BD Bioscience), IL-10R (1B1.3a, Biolegend), CD11b (M1/70, BD Bioscience), Gr1 (RB6-8C5, Biolegend), and anti-idiotypic CEA-Fc (Sorrento Therapeutics). For human CAR-T cells, cells were stained for CFSE, CD25 (BD Bioscience, 560989) and CD69 (Invitrogen, 25069941). For intracellular staining, the BD Bioscience Fixation/Permeabilization kit was used. Unstained and isotype controls were used for gating purposes. Cells were collected for analysis using Cytoflex LX (Beckman Coulter) and post-acquisition analysis was performed using FlowJo software (BD Bioscience).

Live imaging

CRLM tumor slices cultures were created as described above. Untransduced control or CAR-T cells were thawed, washed, and stained with CFSE at a concentration of 0.5 mM. One million of the stained control or antigen-specific CAR-T cells were added on top of slices to be treated in culture followed by either control or αIL-10 to the supernatant added to the media, and slices with CAR-T cells and antibody treatment were incubated for 1 day. Treatment 10^5 CAR-T cell resulted in similar but non-significant results compared to 10^6 (Fig. S4). The treated CRLM tumor slices were transferred into fresh media in a 48 well plate with 10 mg/mL Alexa 647 EpCAM antibody to stain tumor cells (Biolegend, San Diego, CA) and SR-FLICA reagent to stain apoptotic cells per the manufacturer's instructions (ImmunoChemistry Technologies, Bloomington, MN) and were incubated for 3 hours at 37°C for staining. The slices were then stained for nuclei using 10 mg/mL Hoechst 33342 (ImmunoChemistry Technologies) for 10 min. The slices were washed twice with PBS and returned with their original media containing the CAR-T cell and antibody treatment to an 8-well culture slide (Ibidi USA, Fitchburg, WI) for imaging. Tissue culture conditions were maintained as close to cell culture incubator as possible while imaging was performed, by using a heated covered stage (PeCon,

Erbach, Germany) to 37°C while flushing warmed, humidified CO_2 through the enclosure. The slices were imaged using a Leica SP8X confocal microscope (Leica Microsystems, Wetzlar, Germany) at 20x magnification. For each treated slice, images were collected for 1 hour at three different positions throughout the slice with a z-stack of 20 μm. One slice was used per treatment group per patient given imaging constraints with random sequential slices assigned to each treatment group.

In an experiment blocking the IL-10R on CAR-T, the CAR-T were thawed and incubated overnight in tissue culture media with 20 mg/mL of either control or αIL-10R. The CAR-T cells were washed with PBS, spun, and resuspended in media. They were stained with CFSE and placed directly into the CRLM tissue slice culture media without additional treatment or antibody blockade. After 1 day, slices were stained and imaged as above.

No slices or data were excluded from analysis. Treatment groups were unable to be blinded for imaging or analysis. Image processing and data analysis were performed on Leica LAS X software (Leica Microsystems, Wetzlar, Germany) and Imaris software (Bitplane USA, Concord, MA). The data and images obtained were visualized using a maximum intensity projection of the 20 μm z-stack. The $EpCAM^+$, $CFSE^+$, $SR\text{-}FLICA^+$ cells were counted manually. All cells observed throughout the time imaged at each position in each slice were counted.

Bulk RNA sequencing

CRLM slice culture treated with either untransduced or anti-CEA CAR-T cells either with or without IL-10 blocking antibody as described above. RNA was extracted from slices using Qiagen RNEasy kit

per the manufacturer's instructions. Samples were submitted to the Northwest Genomics Center for NGS sequencing.

Single cell RNA sequencing (scRNAseq)

Tumor slice dissociation

Tumor slices were dissociated using the MACS Tumor Dissociation Kit (MiltenyiBiotec, Auburn, CA) according to the manufacturer's "dissociation of soft tumors" protocol. Prior to dissociation, an enzyme mix was prepared by adding fresh aliquots of enzymes A, R and H with the recommended volumes to RPMI 1640 medium in a sterile "gentleMACS C" tube. The initial mechanical dissociation step was performed by running the "h_tumor_01 program." Slices were then incubated at 37°C for 20-30 minutes under continuous rotation using the MACSmix Tube Rotator followed by a second mechanical dissociation step, "h_tumor_02 program". Slices were then visually evaluated and tubes were incubated for an additional 20 minutes at 37◦ C if undissociated tissue was visible. Slice tissue was further dissociated by running the "h_tumor_03" program. After a short spin, the cell suspension was passed through a 70 µm MACS SmartStrainer. The strainer was washed with a solution of 0.04% (w/v) molecular biology grade BSA (Gemini Bio Products) in PBS. Finally, the cell suspension was strained through a 30 µm MACS SmartStrainer. Cells were spun down at 300×g and supernatant was removed.

Removal of dead cells from single cell suspension

Dead cells were removed from single cell suspensions with the Dead Cell Removal kit (MiltenyiBiotec) and according to the Dead Cell Removal Rev B protocol (10X Genomics, Pleasanton, CA). Cells were resuspended in Dead Cell Removal MicroBeads and incubated for 15 minutes at room temperature. Meanwhile, an MS column (MiltenyiBiotec) was rinsed with binding buffer. The cell suspension was then diluted and applied to the MS column on a MiniMACS separator. The effluent containing the live

single cells in binding buffer was collected into sterile tubes. The binding buffer was then replaced with PBS containing 0.04% BSA through two wash steps. Wide-bore pipette tips (Rainin, Columbus, OH) were used to ensure minimum damage to single cells.

Single cell suspension preparation for 10X Genomics Single Cell experiments

The cell suspension was mixed with Trypan Blue, quantified with a Countess Automated Cell Counter (Life Technologies, Waltham, MA), and confirmed with a hemocytometer. Based on the cell concentration and the targeted cell recovery, the corresponding volume of cell suspension was mixed with nuclease free water and loaded on the Chromium Controller according to the Chromium Single Cell 3' Reagent Kits v2 protocol (10X Genomics). On average, 17,000 cells in 33.8 µl were loaded into each Chromium droplet generation microfluidic device to target the maximum cell recovery number of 10,000.

10X Droplet Sequencing

Cellular suspensions were loaded on a Chromium instrument (10X Genomics, San Francisco, CA) to generate single-cell Gelbead-In-EMulsion (GEM) droplets. Reverse transcription (RT) was performed in a C1000 Touch thermocycler (Biorad, Hercules, CA). After RT, GEMs were harvested and the cDNAs were amplified and cleaned with SPRIselect Reagent Kit (Beckman Coulter, Brea, CA). Indexed sequencing libraries were constructed using the Chromium Single-Cell 3' Library Kit (version 2) for enzymatic fragmentation, end-repair, A-tailing, adapter ligation, ligation cleanup, sample index PCR, and PCR cleanup. The barcoded sequencing libraries were quantified by quantitative PCR using the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA). Sequencing libraries were loaded on a NextSeq500 (Illumina, San Diego, CA) and run 150 cycles (26 bp for Read 1 and 124 bp for Read 2). Two indexed (multiplexed) libraries (i.e. Control, anti-IL-10) were loaded on to one flowcell to obtain a sequencing depth of approximately 100,000 reads per cell. Reads were aligned to

the human genome (GRCh38) and quantified using the Cell Ranger (version 2.0, http://cf.10xgenomics.com/releases/cell-exp/ cellranger-2.0.0.tar.gz). Poor-quality cells/GEMs, defined as those expressing a high proportion (>10%) of mitochondrial genes or a low number of total genes (<200) were discarded. Unique transcripts were identified by 10X unique molecular indexes (UMIs). UMI counts were normalized by adjusting for library size and dividing by the median count of each cell, as implemented by the Cell Ranger R kit (version 2.0, http://cf.10xgenomics.com/supp/cell-exp/¬rkit-install-2.0.0.R).

Cell type classification and clustering

Sample UMI data were aggregated and the gene-cell matrix was normalized to give each gene a mean of 0 and standard deviation of 1. Principal Component Analysis was performed, and the top 30 components used to calculate a Uniform Manifold Approximation and Projection (UMAP) for visualization of all single cells in a two-dimensional space. Cells were clustered by the Leiden algorithm (26) as implemented with the SCANPY toolkit (27).

Table S1 – treatment antibodies

Antibody	**Manufacturer**	**Catalog no.**
Monoclonal IgG	BD Biosciences	#553447
Anti-IL-10	Biolegend	#501407
Anti-PD-1	BD Biosciences	#562138
Anti-MHC I	BD Biosciences	#555551
Anti-MHC II	BD Biosciences	#555557

Table S2 – ISH probes, mIHC antibodies, and live fluorescent imaging antibodies**Antibody**	**Fluorophore**	**Manufacturer**	**Catalog no.**
IFNG	Opal 650	Advanced Cell Diagnostics	#550478-C3
IL10RA	Opal 570	Advanced Cell Diagnostics	#498058
IL10	Opal 650	Advanced Cell Diagnostics	#602058-C2
EpCAM	Opal 690	Biolegend	32402
HLA-DR	Opal 620	Dako	M0746
CD3	Opal 570	ThermoFisher	RM9107
CD163	Opal 540	BioSB	BSB3276
CD68	Opal 540	Dako	M0876
EpCAM	Alexa 647	Biolegend	324212
Poly caspases	SR-FLICA	ImmunoChemistry Technologies	916

Table S3 – IHC antibodies

Antibody	**Manufacturer**	**Catalog no.**
cleaved-Caspase-3	Cell Signal Technologies	#9661
CD163	Abcam	ab189915
HLA-DR	Dako	M0746
CD4	Dako	M7310
CD8	Dako	M7103

Table S4 – flow cytometry antibodies

Antibody	**Manufacturer**	**Catalog no.**
CD3	BD Bioscience	17A2
IL-10	BD Bioscience	JES5-16E3
IL-10R	Biolegend	1B1.3a
CD11b	BD Bioscience	M1/70
Gr1	Biolegend	RB6-8C5
anti-idiotypic CEA-Fc	Sorrento Therapeutics	
CD69	Invitrogen	25069941
CD25	BD Bioscience	560989