Supplementary Materials for

TCR-Vγδ usage distinguishes pro-tumor from anti-tumor intestinal γδ T cell subsets

Bernardo S. Reis*, Patrick W. Darcy, Iasha Z. Khan, Christine S. Moon, Adam E. Kornberg, Vanessa S. Schneider, Yelina Alvarez, Olawale Eleso, Caixia Zhu, Marina Schernthanner, Ainsley Lockhart, Aubrey Reed, Juliana Bortolatto, Tiago B. R. Castro, Angelina M. Bilate, Sergei Grivennikov, Arnold S. Han, Daniel Mucida*

* correspondence to: breis@rockefeller.edu and mucida@rockefeller.edu

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Other Supplementary Materials for this manuscript includes the following:

Material and Methods

Human Specimens. Tumors and adjacent normal colon tissues were collected with informed consent from the patients diagnosed with colorectal adenocarcinoma at Columbia University Irving Medical Center, as approved by the Columbia University Institutional Review Board (IRB) (AAAR1384) and Rockefeller University IRB (YAL1027). All protocols were performed in accordance with the guidelines provided by the IRB. All patients were treatment naïve at the time of sample acquisition.

Biopsy Sample Processing. Fresh tissue was collected the same day of the surgery and the tissue was dissociated into single-cell suspensions with enzymatic digestions using gentleMACS dissociator per manufacturer's protocol (Miltenyi Biotec). Single cell suspensions were cryopreserved (40% FBS 10% DMSO) and stored in liquid nitrogen.

Mice. C57BL/6J control (000664), *Trdc*^{GFP} (016941), *Trdc*$^{-/-}$ (002120), *Trdc*^{CreER} (031679), *Scl2a1*$^{fl/fl}$ (031871), *Rorc*$^{fl/fl}$ (008771), *Cdx2*^{CreER} (022390), Rosa26^{dTomato} (007914) were purchased from Jackson Laboratories and kept in our facility. APC$^{fl/fl}$ mice were provided by S. Grivennikov (C. Sinai) (*14, 23, 24*). E8I^{CRE} mice were provided by Ichicro Taniuchi (RIKEN, Japan). Vγ4$^{-/-}$, Vγ6$^{-/-}$ and Vγ7$^{-/-}$ mice were developed by mutating *Trgv4*, *Trgv6*, and *Trgv7* genes, respectively, using CRISPR/Cas9 genome editing as described below. CRISPR mutant mice were backcrossed at least 5 times to B6 mice prior to use in experiments. Heterozygous or wild-type homozygous littermates were used as controls as indicated in figure legends. Mice were bred within our facility to obtain strains described and were 7-12 weeks of age for all experiments. To minimize microbiome fluctuations in the AOM-DSS model, genetically modified and wild-type control animals were co-housed from birth. Male and female mice were used in all other experiments, in similar distribution, unless otherwise stated in the figure legends. Estrous cycle was not controlled for in female mice. Animal care and experimentation were consistent with NIH guidelines and

approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University. All animals were kept in specific pathogen free (SPF) conditions.

Generation of Vγ specific knockout by CRISPR-Cas9 gene editing. For the generation of specific Vγ knockout animals, gRNA targeting specific TRVG genes were designed and synthetized as sgRNA by IDT. sgRNA and Cas9 protein were injected in the pronucleus of 1-cell-stage fertilized C57BL/6 embryos. Injected embryos were implanted into pseudo-pregnant foster Swiss animals and mutant offspring were selected by specific PCR genotyping (**Table S2**) and Sanger DNA sequencing. Mutants were backcrossed to C57BL/6 animals for at least 5 generations (as indicated in figure legends). For TRVG4 gene targeting, sgRNA sequence GUGUAACCAUACACUGGUACCGG was used. For TRVG6 gene targeting, sgRNA AGCCCGAUGCAUACAUACACUGG and for TRVG7 gene targeting sgRNA sequence ACUGGUACCGAUUCCAGAAAGGG was used. sgRNA and Cas9 protein were purchased from Integrated DNA Technologies (IDT). Guides with a MIT score higher than 89 were picked on UCSC Genome Browser. Animals were kept in our facility under the Rockefeller University IACUC protocol.

Colorectal cancer (CRC) models

AOM-DSS model. Mice were injected with 12.5mg/kg of Azoxymethane (AOM) 1 day prior to 3 consecutive oral exposures of dextran sodium sulfate (DSS, 2% in drinking water) for 1 week with 2 weeks interval between exposures. Animals were sacrificed 12 weeks after initial AOM injection. Tumors were counted and measured at the time of experiment termination.

APC model. *Cdx2*^{CreER}:APC$^{fl/fl}$ (*iCdx2*$^{\Delta APC}$) mice were injected twice with 0.8 mg of tamoxifen (Sigma) 4mg/ml in corn oil (Sigma), two days apart. Four weeks after the first injection, mice were sacrificed, and tumors were measured and counted. Animals were monitored for weight loss and sickness behavior and the experiment was terminated when weight loss was greater than 20% of initial weight.

Tamoxifen treatment regimen in AOM-DSS model. Tamoxifen (Sigma) was administrated by intra-peritoneal injections, twice a week, of 0.8mg of tamoxifen diluted 4 mg/mL in corn oil (Sigma). For early gene depletion, tamoxifen injections started 1 week before AOM administration and continued until week 3 of AOM-DSS CRC model. For late gene targeting, tamoxifen injections started 1 week after second round of DSS and continued until the week 12 of the AOM-DSS CRC model.

Antibiotic and EdU administration. *iCdx2*$^{\Delta APC}$ mice were treated for one week with broad spectrum antibiotics consisting of 1mg/ml ampicillin, 1mg/ml neomycin, 0.5mg/ml vancomycin, 0.5mg/ml metronidazole and 10g/ml sucralose in the drinking water during the final week of the experiment. EdU was also added to the antibiotic containing water at 1mg/ml. As a control, 10mg/ml sucralose solution containing 1mg/ml EdU was used. EdU detection was performed using the Click-iT™ Plus EdU Flow Cytometery Assay kit (Thermo Fisher Scientific, C10632), according to manufacturer's instructions.

***In vivo* antibody administration.** For depletion of Vγ1^{+} T cells, 200μg of anti-Vγ1 antibody 2.11 (Bio X-Cell) was injected intraperitoneally twice a week 1 week before and for the first 2 weeks of AOM-DSS model. For depletion of Vγ4^{+} T cells, 200μg of anti-Vγ4 antibody UC3-10A6 (Bio X-Cell) was injected intraperitoneally twice a week for the final 6 weeks of AOM-DSS model. For TCRγδ blockage, 400μg of anti-TCRγδ antibody UC7-13D5 (Bio X-Cell) was injected intraperitoneally twice a week at indicated periods of APC loss model.

Isolation of intestinal and tumor infiltrating cells. Intraepithelial lymphocytes were isolated as previously described (*46*). Briefly, large intestines were harvested and washed in PBS and 1mM dithiothreitol (DTT) followed by 30 mM EDTA. Intraepithelial cells were recovered from the supernatant of DTT and EDTA washes. Tumor infiltrating cells were obtained after collagenase digestion of the tissue. Dissected tumor was minced and incubated with 50mg/ml of collagenase VIII, 200mg/ml of DNAse in RPMI for 30 minutes. Mononuclear cells were isolated by gradient centrifugation using Percoll. Single-cell

suspensions were then stained with fluorescently labeled antibodies for 25min at 4°C prior to flow cytometry analysis and/or cell sorting.

Flow cytometry. Cells were stained for anti-CD45 (BD 561487 clone 30-F11), anti-TCRβ (BioLegend 109220 clone H57-597) anti-TCRγδ (eBioscience 11-5711-85 clone GL-3), anti-CD8α, (eBioscience 56-0081-82 clone 53-6.7), anti-CD4 (BioLegend 100552 clone RM4-5), anti-PD-1 (BioLegend 135219 clone 29F.1A12), anti-Vγ1 (BioLegend 141109 clone 2.11), anti-Vγ4 (eBioscience 25-5828-82 clone UC3-10A6), anti-Vγ6 (clone 17D1, kindly donated by Yasmin Belkaid, Ph.D.), anti-Vγ7 (clone GL1.7, kindly donated by Rebecca O'Brien, Ph.D.), anti-CD107 (eBioscience 46-1071-82 clone 1D4B), anti-IFN-γ (eBioscience 45-7311-82 clone XMG1.2), anti-IL-17 (BD 559502 clone TC11-18H10), anti-CD11b (eBioscience 47-0112-82 clone M1/70). For anti-Vγ6 staining, cells were first stained with anti-TCRγδ (clone GL-3) for 30 minutes prior anti-Vγ6 17D1 antibody staining. For cytokine profiling, tissue isolated cells were stimulated ex-vivo with 1mg/ml of phorbol myristate acetate (PMA) and 1mg/ml of Ionomycin in the presence of 10mg/ml of Brefeldin for 4 hours prior staining.

Single-cell RNA sequencing. Cryopreserved single cell suspensions of human colon biopsies were thawed at 37 C, washed with RPMI, and rested in complete RPMI in 96-well plate for 1 hour. The cells were subsequently incubated with PMA/Ionomycin for 3 hours, washed, blocked in Fc receptor block (TruStain FcX, Biolegend), and tagged with cell hashing antibody. Conjugation of purified antibodies for cell hashing was performed as described by the Technology Innovation Lab at the New York Genome Center (https://cite-seq.com/protocol/). The list of antibodies, clones, and barcodes are available in **Table S3**. The hashtagged samples were then pooled and stained for FACS sorting with anti-CD3 (clone OKT3, BioLegend) APC, anti-TCRγδ (clone 5A6.E9, Thermo Fisher) PE, and anti-TCRαβ (clone IP26, BioLegend) Alexa Fluor 488. Samples were subsequently stained with Sytox Blue (Thermo Fisher) prior to acquisition to determine cell viability. Viable T cells were sorted in FACS Aria II by gating SytoxBlue$^-$

$CD3^+TCR\gamma\delta^+TCR\alpha\beta^-$ cells and subsequently transferred into RPMI with 10% FBS at 4C. FACS-sorted $CD3^+$ $TCR\gamma\delta^+$ cells were loaded onto the Chromium 10x Genomics v1.1 platform (10X Genomics) as described in the manufacturer's protocol. Single cell 5' transcript and TCRγδ libraries were prepared using Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 with Feature Barcode Technology for Cell Surface Protein (CG000228). Following cDNA amplification, cell hashing additive oligos were added to amplify cell hashtag oligos. The products were separated using SPRIselect Beads (Beckman Coulter). Cell hashtag library generation was performed as described by the Technology Innovation Lab at the New York Genome Center (https://cite-seq.com/protocol/). TCRγδ libraries were prepared as described in Mimitou *et al.,* 2019. cDNA libraries were quantified using Agilent High Sensitivity D5000 ScreenTape (Agilent, no. 5067-5592). Libraries were sequenced using NovaSeq 6000 platform.

Data Processing of Single-Cell RNA-Seq and Single Cell TCR-Seq Libraries. Raw fastq files derived from our RNA-seq libraries were processed with cellranger count (v6.0.2) using the 10x Genomics prebuilt human reference (GRCh38 - GENCODE v32/Ensembl 98). The count matrix was processed using Seurat (v4.0.4) (*47*). Quality control was performed by removing cells with high (> 10%) mitochondrial-derived UMIs. The assembly, alignment, and annotation of TCR sequences were performed using the Cellranger vdj workflow from 10x Genomics and the prebuild human vdj reference (GRCh38 - Ensembl5.0.0). The assignment of gamma/delta T-cell clones was performed using the clonality package (https://github.com/victoraLab/clonality). Paired TCR clones were defined by identical V, (D), J genes, and CDR3 length and nucleotide sequence for delta and gamma chains. Cells with only one successfully TRG or TRD chain sequenced were assigned as part of a paired TCR core if its DNA sequence matched its correspondent chain completely (sticky parameter).

Single Cell RNA-Seq Normalization and Statistical Analysis. The raw UMI counts were normalized using the SCTransform function available in the Seurat (v4.0.4) package (*48*). The top 3000 variable genes were first used for dimensional reduction by PCA using the scaled data. The first 30 principal components

were further used for visualization using the Manifold Approximation and Projection (UMAP) and cell clustering (*47, 48*). The Wilcoxon rank-sum test detected differentially expressed genes by comparing each cluster against all cells. Genes with adjusted p-values of 0.05 or less were considered for downstream analysis. Finally, we scored each cell based on gene sets for the S and G2M phases using the CellCycleScoring function within the Seurat package (*49*). Cells that did not score in at least one set were marked as in the G1 phase. GSEA analyses were performed using the fgsea package for R (*50*) and the Molecular Signatures Database (MSigDB - v7.5.1)[PMID: 16199517]. In short, for each condition or cluster, genes were ranked using their Log2FoldChanges, and enriched pathways were considered significant when having adjusted p-values of 0.05 or less. Heatmap was constructed using the ComplexHeatmap package (*51*) with the Pearson residuals matrix obtained by GetResidual function from Seurat.

Generation of Circos Plots and Alluvial Diagrams. TCR sharing (clonal overlap) was visualized using Circos to create circular plots aesthetics (*52*). Each ideogram denotes a cell population as indicated in the figures, with bands representing clonal distribution. Alluvial diagrams were generated with the ggalluvial package for R (*53*).

Bulk mRNA-Seq Library Preparation. Sorted cells (300-800 cells) were lysed in a guanidine thiocyanate buffer (TCL buffer, QIAGEN) supplemented with 1% β-mercaptoethanol. RNA was isolated by solid-phase reversible immobilization bead cleanup using RNAClean XP beads (Agentcourt, A63987), reverse transcribed, and amplified as described (*54*). Uniquely barcoded libraries were prepared using Nextera XT kit (Illumina) following manufacturer's instructions. Sequencing was performed on an Illumina NextSeq500.

Bulk RNA-Seq Analysis. Raw fastq files were aligned and quantified with STAR (v. 2.7.10a) by using the mouse genome (mm39) and the mouse transcriptome (gencode M29) (*55*). Next, the count matrix was

imported to the R environment and processed by the DESeq2 package (v. 1.34) pipeline (*56*). Briefly, genes from samples expressing less than ten reads were pre-filtered. Gene expression among samples was normalized by applying a negative binomial distribution model. The Wald test was employed to determine differential gene expression between conditions. Genes containing adjusted pvalues (FDR) less than 0.1 were considered for downstream analysis, and log2 fold changes were shrunk using the ashr algorithm (*57*).

Single-Cell TCR Sequencing. Single cells were index-sorted using a FACS Aria into 96-well plates containing 5 μL of lysis buffer (TCL buffer, QIAGEN 1031576) supplemented with 1% β-mercaptoethanol and frozen in −80°C prior to RT-PCR. RNA and RT-PCRs for TCRγ and TCRδ were prepared using specific primers (**Table S4**). PCR products for TCRγ and TCRδ were multiplexed with barcodes and submitted for MiSeq sequencing (*58*) using True Seq Nano kit (Illumina). Fastq files were de-multiplexed and paired-end reads were assembled at their overlapping region using PANDASEQ (*59*) and FASTAX toolkit. De-multiplexed and collapsed reads were assigned to wells according to barcodes. Fasta files were aligned and analyzed on IMGT (http://imgt.org/HighV-QUEST) (*60*). Cells with identical TCRγ and TCRδ CDR3 amino acid sequences were considered as the same clones. Clonality was assigned based on paired TCRγδ per mouse. Only in frame junction sequences of TCRγδ were included in the analysis.

FocusClear and cell localization on villus-crypt axis. Tissue clearing was performed according to FocusClear manufacturer's (Celexplorer Labs. Co.) instructions. Briefly, $E8_I$ (*Cd8a* enhancer I)^{Cre}:*Rosa26*$^{fsf\text{-}dTomato}$ ($E8_I^{dTomato}$):*Trdc*^{GFP} double-reporter mice were injected with Hoechst dye (blue) for visualization of epithelial cell nuclei. After 15-30 min, mice were sacrificed by cervical dislocation and segments of the small intestine were removed, washed (intact) and fixed in 4% PFA at gentle agitation for 2h at RT. After fixation, samples were washed in DPBS and placed in FocusClear solution for approximately 15 min. at room temperature. Once visual confirmation of clearance was obtained, samples were mounted in 3D printed slides with MountClear, sealed and imaged using an inverted LSM 880 NLO

laser scanning confocal and multiphoton microscope (Zeiss). As this protocol maintains native GFP and dTomato, and introduced Hoechst fluorescence, no antibody staining was necessary. Cell X and Y coordinates were obtained using *Imaris* (Bitplane AG) software, as described before (*3*). Multiple technical replicates per sample (segment) were obtained.

Intravital imaging. Tamoxifen-treated iCdx2Δ^{APC}_*Trdc*^{GFP} reporter mice were anesthetized by continuous administration 1% of isofluorane and 1L per minute of oxygen mixture while imaging was performed. Mice were injected with Hoechst dye (blue) for visualization of epithelial cell nuclei. 10 min following induction of anesthesia, mice were placed on a custom platform heated to 37°C. Upon loss of recoil to paw compression, a small incision was made in the abdomen. The cecum and the proximal colon were located and placed onto a raised block of thermal paste covered with a wetted wipe paper. A coverslip was placed on top of the loop to immobilize the intestine. The platform was then transferred to the FV1000MPE Twin upright multiphoton system (Olympus) heated stage. Time-lapse was ± 30sec with a total acquisition time of 15 min. A complete Z stack (40 to 60 μm) was made during each acquisition. *Imaris* (Bitplane AG) software was used for cell identification and tracking, using the "*Spots"* auto-regressive tracking algorithm as described by the manufacturer. The scoring of IEL behavior (speed) was entirely computational and unbiased.

Statistical Analyses. Statistical analyses were carried out using GraphPad Prism v.9. Flow cytometry analyses were carried out using FlowJo software. Data in graphs show mean ± SEM and P values < 0.05 were considered significant. Statistical analyses were performed using ordinary one-way ANOVA test with Bonferroni's multiple comparison test and two-tail t-test, as indicated in the figure legends. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. GraphPadPrism v.9 was used for graphs and statistical analysis, and Adobe Illustrator 2020 was used to assemble and edit figures.

Movie S1. iCdx2$^{\Delta APC}$ animals were i.p. injected twice with 0.8mg of Tamoxifen 2 days apart and subjected to intravital imaging of colonic γδ T cells at indicated time points. Two weeks pos Tamoxifen injection, animals were treated with 400μg of anti-TCRγδ blocking antibody (UC7-13D5) three times a week and subjected to intravital imaging of colonic γδ T cells.

Movie S2. iCdx2$^{\Delta APC}$ animals were i.p. injected twice with 0.8mg of Tamoxifen 2 days apart and treated with 400μg of anti-TCRγδ blocking antibody (UC7-13D5) three times a week. Animals were subjected to intravital imaging of cecal γδ T cells three weeks after initial Tamoxifen treatment.

Data S1. single-cell TCR sequencing analysis (scTCR-seq) of sorted γδ T cells from tumor and adjacent nontumor areas of *iCdx2*$^{\Delta APC}$ mice 4 weeks after tamoxifen injection.

Data S2. single-cell TCR sequencing analysis (scTCR-seq) of sorted γδ T cells from tumor and adjacent nontumor areas of wild type B6 mice subjected to AOM-DSS model.

Data S2. single-cell TCR sequencing analysis (scTCR-seq) of sorted γδ T cells from tumor and adjacent nontumor areas of Vγ4$^{-/-}$ and littermate control mice subjected to AOM-DSS model.

Table S1. CRC patient cohort. Tumor information from accessed patients and expected γδ T cell numbers from non-tumor and tumor areas prior sorting.

Table S1

ID	Dem	Type	Tumor Grade	Location	Tumor Invasion	Sample Info	% γδ cells/CD3+	# γδ cells/tube	# Tubes	Expected γδ	Total cells	Sorted
337	60M	Microsatellite +	G2-3 Adenocarcinoma	Ascending	muscularis propria into pericolorectal tissue (T3)–pN1a	Nomal	40%	790	2	1580	10M	YES
						Tumor	10%	170	4	680	20M	
859	74F		G2 Adenocarcinoma	Ascending	Submucosa (T1)–pN0	Nomal	Not assessed	Not assessed	4	Not assessed	40M	YES
						Tumor	5%	350	2	700	200K	
989	59F		G2 Adenocarcinoma	Transverse	Submucosa (T1)–pN0	Nomal	Not assessed	Not assessed	4	Not assessed	24M	YES
						Tumor	1%	50	2	100	1.6M	
1	75F	KRAS Q61H	G2 Adenocarcinoma	Sigmoid	muscularis propria into pericolorectal tissue (T3)–pN0	Nomal	0	0	4	0	20M	NO
						Tumor	0	0	4	0	20M	
391	48F	FBXW7 E471fs	G2 Adenocarcinoma	Rectosigmoid	visceral peritoneum (including tumor continuous with serosal surface through area of inflammation) (T4a)–pN1b	Nomal	1%	70	5	350	15M	YES
						Tumor	1%	100	6	600	30M	
507	53F	FBXW7 S582L	G2 Adenocarcinoma	Sigmoid	muscularis propria (T2)–pN1b	Nomal	Not assessed	Not assessed	3	Not assessed	300K	NO
						Tumor	1%	10	2	20	1.4M	
633	79F		G2-3 Adenocarcinoma	Sigmoid	visceral peritoneum (including tumor continuous with serosal surface through area of inflammation) (T4a)–pN1c	Nomal	1%	10	1	10	4M	YES
						Tumor	1%	120	3	360	12M	

Table S2. Genotype primers for *Trgv*-specific knockout mice.

Table S2

Geno	FWD-primer	REV-primer	Band size
Vg4-WT	TAGGAGTGTAACCATACACTGGT	AGATGCTGTGCTTTAGCCGT	223bp
Vg4-Mut	CCTGCCAGTCATTCATGGGT	GGTTTCTGCCGATGAAAACTTTAC	133bp
Vg6-WT	TACCAAGAGAAGCCAGGCCAG	CCTAGGTGTCTGTGCAGGTG	266bp
Vg6-Mut	CAGAGGGAAGCAGTCTCACG	TTGGAGACGCTGGCCTTGGTA	113bp
Vg7-WT	ACTGGTACCGATTCCAGAAAG	GAGGGCAAGGCTGAGTCATT	222bp
Vg7-Mut	AGCCATGCTGATGAGTTGCT	CTCTGGGCCCAGTGGATGTAA	141bp

Table S3. Hashtag antibodies used in sorted γδ T cells from CRC patients.

Table S3

Antibody	Clone	Manufacturer	Isotype	Hash ID	Barcode	Sample
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash1	gaagatacacgt	337N
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash2	gtgagatatcac	337T
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash3	gaataacagccc	859N
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash4	caaagcgtatct	859T
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash5	gatgtggttaga	989N
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash6	agacgcggattg	989T
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash7	gacaggactttg	391N
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash34	tgctcaaaatgc	391T
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash35	gtctgtgacgta	633N
anti-human β2-microglobulin	2M2	BioLegend	Mouse IgG1, κ	hash36	aggtcgacgaca	633T

Table S4. Primer list for TCRγ and TCRδ amplification and sequencing.

Table S4

V segments	Forward (1st PCR)	Constant	Reverse (1st PCR)
Vg1/2/3	GCAGCTGGAGCAAACTGAA	TRGC1/2	GGGAGTCCAGGATAGTATTGCC
Vg4	GGAAGTTGGAGCAACCTGAA	TRGC4	GGGATTCCAGAATCTTTTCACCA
Vg5	TCTCCTTTACCCGAAGACCA		
Vg6	GAGGGAAGCAGTCTCACGTC		
Vg7	ACCAAGCTAGAGGGGTCCT		

V segments	Forward (2nd PCR)	Constant	Reverse (2nd PCR)
Vg1/2/3	ATCGGTCACCAGAGAGACAG	TRGC1/2	GAAAATAGTGGGCTTGGGGGA
Vg4	CCAAGAGATGAGACTGCACAA	TRGC4	CAGCAGCAGAAGGAAGGAAAAT
Vg5	CAAACAAGACGGTGCACATAA		
Vg6/Vg7	TACATMCACTGGTACCRA		

adaptor 2nd PCR	CCAGGGTTTTCCCAGTCACGAC		
V segments	Forward (3rd PCR)	Constant	Reverse (3rd PCR) + barcodes
Vg1/2/3	CCAGGGTTTTCCCAGTCACGAC ATCGGTCACCAGAGAGACAC	TRGC1/2	CTTGGGGGAAATGTCTGCAT
Vg4	CAGGGTTTTCCCAGTCACGAC CCAAGAGATGAGACTGCACA	TRGC4	GCTTGGGAGAAAAGTCTGAGT
Vg5	CAGGGTTTTCCCAGTCACGAC CAAACAAGACGGTGCACATAA		
Vg6/Vg7	CCAGGGTTTTCCCAGTCACGAC TACATMCACTGGTACCRA		

V segments	Forward (1st PCR)	Constant	Reverse (1st PCR)
Vd1	GAAGTCACCATGAGCTGCAA		AGCAGGGTCGAATTCCACAAT
Vd2-1/2-2	ACCCTGGACTGCACCTATG		
Vd4	CTGCCTCCTTCTACTGCAC		
Vd5	CGGCACTGAAGTAACACTGC		
Vd6-1/6-2/6d-1/6d-2	GGMGAAGAASTCACCCTG		
Vd7	AACGCAGAGCTGCAGTGTAA	TRDC	
Vd8	AGGAGAGACCGCAATTCTGA		
Vd9	GTCAAGTGGCCCTCTCAGAA		
Vd10	ACCGGTTCTGCTCTGAGATG		
Vd11	TGGAGAAGACAACGGTGACA		
Vd12	AAGGAGAAACGGTGCACTTG		

V segments	Forward (2nd PCR)	Constant	Reverse (2nd PCR)
Vd1	CTGGTATAGGCAGGGGGATGA		ATGAAAACAGATGGTTTGGCCG
Vd2-1/2-2	GGCAGGTGACTCTCGTCATT		
Vd4	ACTGCACTGTAACAGGAGGG		
Vd5	CAAAAGGCCAGACAGATCCT		
Vd6-1/6-2/6d-1/6d-2	CACCCTGGACTGTTCATATG		
Vd7	TCGTCAGCCTGTTGTCCAAT	TRDC	
Vd8	TCCCGCTCTCCTGATATCCAT		
Vd9	GCAGTATCCCGGAGAAGGTC		
Vd10	GGGCAGCCTCATCAATCTGT		
Vd11	CAAGCAAACAGCAAGTGGGG		
Vd12	AGGTTCCTCTTCAGGGTCCA		

adaptor 2nd PCR	CCAGGGTTTTCCCAGTCACGAC		
V segments	Forward (2nd PCR)	Constant	Reverse (3rd PCR) + Barcodes
Vd1	GGMGAAGAASTCACCCTG CTGGTATAGGCAGGGGGATGA		AACAGATGGTTTGGCCGGAG
Vd2-1/2-2	GGMGAAGAASTCACCCTG GGCAGGTGACTCTCGTCATT		
Vd4	GGMGAAGAASTCACCCTG ACTGCACTGTAACAGGAGGG		
Vd5	GGMGAAGAASTCACCCTG CAAAAGGCCAGACAGATCCT		
Vd6-1/6-2/6d-1/6d-2	GGMGAAGAASTCACCCTG CACCCTGGACTGTTCATATG		
Vd7	GGMGAAGAASTCACCCTG TCGTCAGCCTGTTGTCCAAT	TRDC	
Vd8	GGMGAAGAASTCACCCTG TCCCGCTCTCCTGATATCCAT		
Vd9	GGMGAAGAASTCACCCTG GCAGTATCCCGGAGAAGGTC		
Vd10	GGMGAAGAASTCACCCTG GGGCAGCCTCATCAATCTGT		
Vd11	GGMGAAGAASTCACCCTG CAAGCAAACAGCAAGTGGGG		
Vd12	GGMGAAGAASTCACCCTG AGGTTCCTCTTCAGGGTCCA		

Figure S1

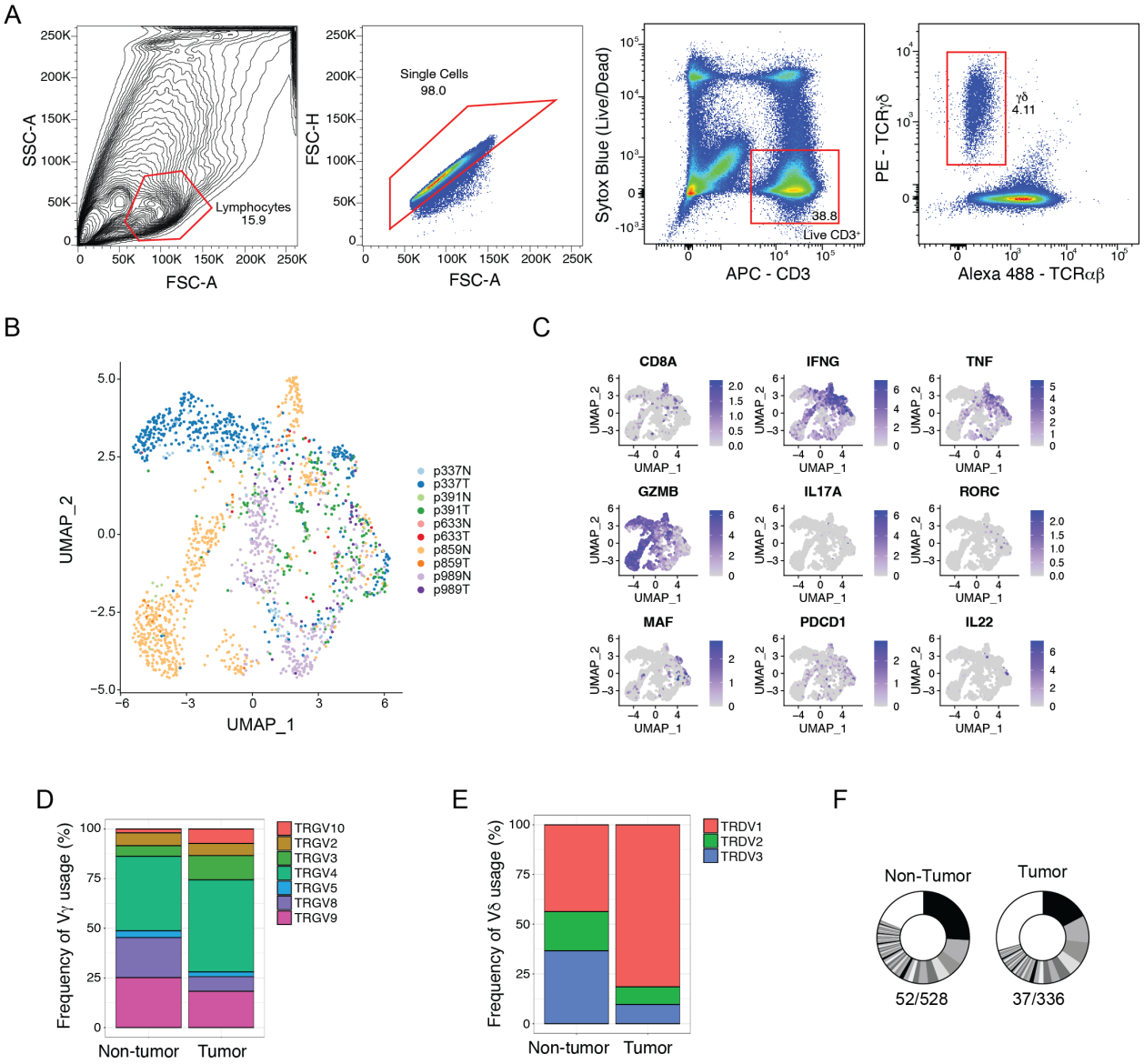
A
SSC-A
FSC-A
Lymphocytes
15.9
FSC-H
Single Cells
98.0
Sytox Blue (Live/Dead)
APC - CD3
38.8
Live CD3+
PE - TCRγδ
Alexa 488 - TCRαβ
γδ
4.11
B
UMAP_2
UMAP_1
p337N
p337T
p391N
p391T
p633N
p633T
p859N
p859T
p989N
p989T
C
CD8A
IFNG
TNF
GZMB
IL17A
RORC
MAF
PDCD1
IL22
D
Frequency of Vγ usage (%)
TRGV10
TRGV2
TRGV3
TRGV4
TRGV5
TRGV8
TRGV9
Non-tumor
Tumor
E
Frequency of Vδ usage (%)
TRDV1
TRDV2
TRDV3
Non-tumor
Tumor
F
Non-Tumor
Tumor
52/528
37/336

Figure S1. Supporting data to Figure 1. (**A**) Gate strategy used for sorting $CD3^{+}SytoxBlue^{-}\gamma\delta^{+}$ cells from both tumor and non-tumor areas of CRC patients for 10X Genomic RNA sequencing. (**B**) UMAP cluster of non-tumor (N) and tumor (T) isolated γδ T cells colored by patient ID. (**C**) UMAP cluster of non-tumor and tumor isolated γδ T cells showing specific gene expression. (**D, E**) Frequency of Vγ (**D**) and Vδ (**E**) usage by TCR sequenced $\gamma\delta^{+}$ T cells from tumor and non-tumor areas. (**F**) Pie chart showing $\gamma\delta^{+}$ T cell clonal expansion in tumor and non-tumor area. Number of expanded clones by the total number of clones sequenced are depicted.

Figure S2

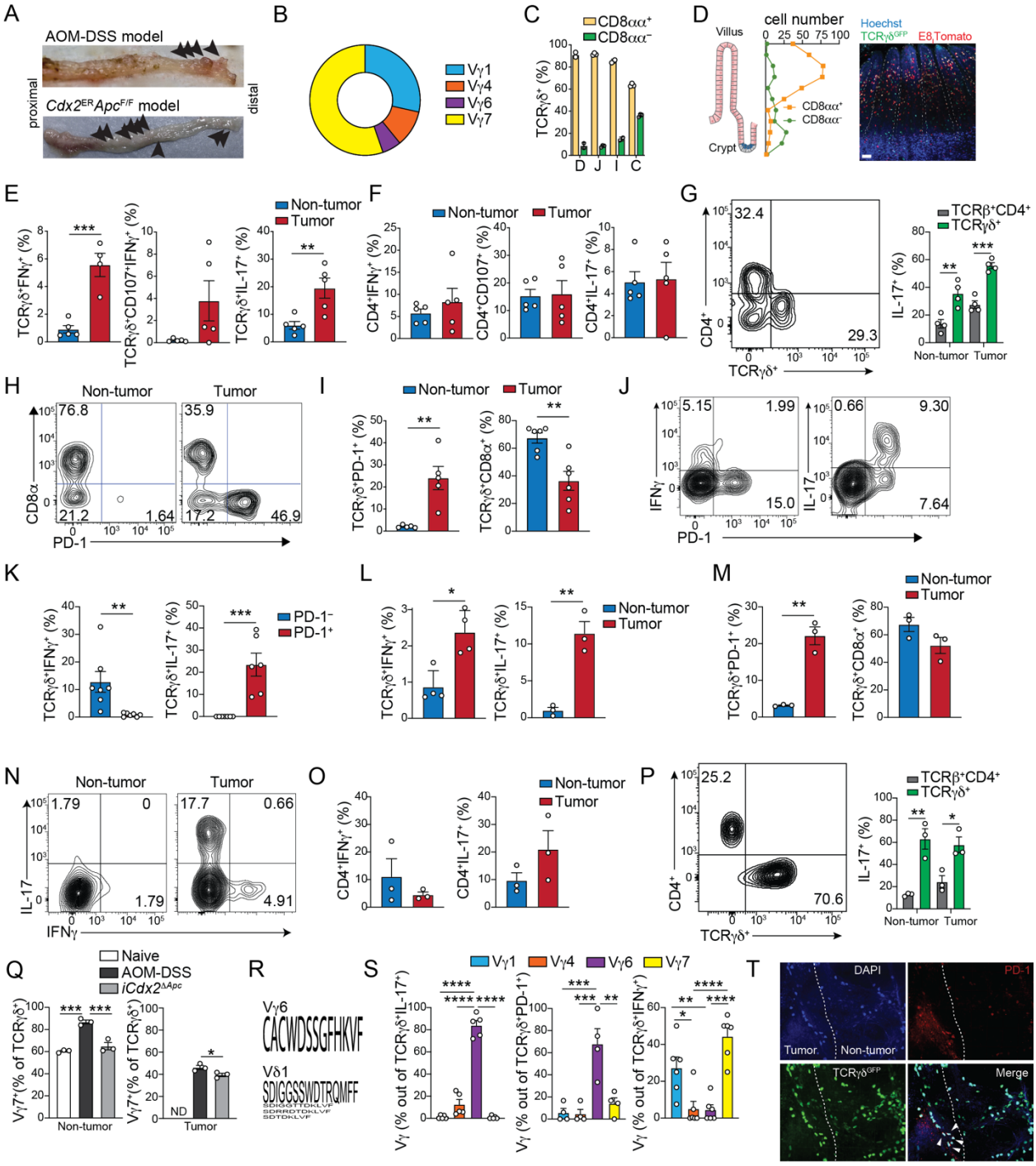
A
AOM-DSS model
proximal
distal
Cdx2ERApcF/F model
B
Vγ1
Vγ4
Vγ6
Vγ7
C
CD8αα+
CD8αα−
TCRγδ+ (%)
D J I C
D
Villus
cell number
0 25 50 75 100
CD8αα+
CD8αα−
Crypt
Hoechst
TCRγδGFP
E8.Tomato
E
Non-tumor
Tumor
TCRγδ+IFNγ+ (%)
TCRγδ+CD107+IFNγ+ (%)
TCRγδ+IL-17+ (%)
F
Non-tumor
Tumor
CD4+IFNγ+ (%)
CD4+CD107+ (%)
CD4+IL-17+ (%)
G
32.4
29.3
CD4+
TCRγδ+
TCRβ+CD4+
TCRγδ+
IL-17+ (%)
Non-tumor
Tumor
H
Non-tumor
Tumor
76.8
35.9
21.2
1.64
17.2
46.9
CD8α
PD-1
I
Non-tumor
Tumor
TCRγδ+PD-1+ (%)
TCRγδ+CD8α+ (%)
J
5.15
1.99
15.0
0.66
9.30
7.64
IFNγ
IL-17
PD-1
K
TCRγδ+IFNγ+ (%)
TCRγδ+IL-17+ (%)
PD-1−
PD-1+
L
Non-tumor
Tumor
TCRγδ+IFNγ+ (%)
TCRγδ+IL-17+ (%)
M
Non-tumor
Tumor
TCRγδ+PD-1+ (%)
TCRγδ+CD8α+ (%)
N
Non-tumor
Tumor
1.79
0
1.79
17.7
0.66
4.91
IL-17
IFNγ
O
Non-tumor
Tumor
CD4+IFNγ+ (%)
CD4+IL-17+ (%)
P
25.2
70.6
CD4+
TCRγδ+
TCRβ+CD4+
TCRγδ+
IL-17+ (%)
Non-tumor
Tumor
Q
Naive
AOM-DSS
iCdx2ΔApc
Vγ7+(% of TCRγδ+)
Non-tumor
ND
Tumor
R
Vγ6
CACWDSSGFHKVF
Vδ1
SDIGGSSWDTRQMFF
SDGGTTDKLVF
SDRRDTDKLVF
SDTDKLVF
S
Vγ1
Vγ4
Vγ6
Vγ7
Vγ (% out of TCRγδ+IL-17+)
Vγ (% out of TCRγδ+PD-1+)
Vγ (% out of TCRγδ+IFNγ+)
T
DAPI
PD-1
Tumor
Non-tumor
TCRγδGFP
Merge

Figure S2. Supporting data to Figure 2. (**A**) Illustrative image of the colon of C57BL/6 mice injected with 12.5mg/kg of Azoxymethane (AOM) 2 days prior to three rounds of 1 week "on" and 2 weeks "off" of 2% Dextran sodium sulfate (DSS) in the drinking water (AOM-DSS model) (top) and tamoxifen-treated iCdx2$^{\Delta APC}$ mice (bottom). Arrows indicate visible tumors (polyps). (**B, C**) Flow cytometry analysis of naïve C57BL/6 mice. (**B**) Frequency of Vγ usage by colonic γδ T cells. (**C**) Frequency of CD8αα^{+} and CD8αα^{-} among TCRγδ^{+} cells from duodenum D, jejunum J, ileum I and colon C. (**D**) Whole-mount imaging of tissue-cleared ileum from E8I^{Tomato}Trdc^{GFP} double reporter mouse at steady-state. Image shows TCRγδ^{+}CD8αα^{+} (Tomato^{+}GFP^{+}) and TCRγδ^{+}CD8αα^{-} (Tomato^{-}GFP+) cells along the villus-crypt axis. Scale bar= 40mm. (**E-K, S**) Flow cytometry analysis of γδ T cells from tumor or non-tumor colonic tissue of C57BL/6 mice subjected to AOM-DSS model. (**E**) Frequency of IFN-γ^{+} (left), CD107+ (center) and IL-17^{+} (right) among TCRγδ^{+} cells. (**F**) Frequency of IFN-γ^{+} (left), CD107^{+} (center) and IL-17^{+} (right) among TCRαβ^{+}CD4^{+} cells. (**G**) Representative dot-plot (left) and frequency of CD4^{+} and TCRγδ^{+} cells among IL-17^{+} cells. (**H, I**) Representative dot-plot and frequency of CD8α^{+} and PD-1^{+} among TCRγδ^{+} cells. (**J, K**) Representative dot-plot and frequency of IFN-γ^{+} and IL-17^{+} among TCRγδ^{+}PD-1^{+} and TCRγδ^{+}PD-1^{-} cells. (**L-P**) Flow cytometry analysis of γδ T cells from tumor or non-tumor colonic tissue of tamoxifen-treated iCdx2$^{\Delta APC}$ mice. (**L**) Frequency of IFN-γ^{+} (left) and IL-17^{+} (right) among TCRγδ^{+} cells. (**M**) Frequency of PD-1^{+} (left) and CD8α^{+} (right) among TCRγδ^{+} cells. (**N**) Representative plot of IFN-γ and IL-17 expression by TCRγδ^{+} cells. (**O**) Frequency of IFN-γ^{+} (left) and IL-17^{+} (right) among TCRαβ^{+}CD4^{+} cells from colonic tumor. (**P**) Representative dot-plot (left) and frequency (right) of CD4^{+} and TCRγδ^{+} cells among IL-17^{+} cells. (Q) Frequency of Vγ7^{+} among TCRγδ^{+} cells from naïve animals, animals subjected to AOM-DSS and tamoxifen treated iCdx2$^{\Delta APC}$ mice. (**R**) Amino acid sequence used by Vγ6Vδ1 expanded (Figure 2F). Font size represents frequency of usage. (**S**) Frequency of Vγ1^{+}, Vγ4^{+}, Vγ6^{+} and Vγ7^{+} among tumor-infiltrating γδ T cells expressing IL-17, PD-1 or IFN-γ. (**S**) Immunofluorescence microscopy analysis of PD-1 expressing TCRγδ^{+} cells on colonic tissue of iCdx2$^{\Delta APC}$ Trdc^{GFP} reporter mice 4 weeks after tamoxifen

administration. Arrows show GFP^+ $PD\text{-}1^+$ double labeled cells. For tissue labeling, samples were incubated with DAPI. (**A-Q**) Data representative from 2 experiments with 3-5 animals per group. (**S**) Data representative from 2 independent experiment. For cytokine staining, cells were stimulated with PMA and Ionomycin. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (**E-G, I, K-M, O, P**) two-tailed t-test; (**Q, S**) One-way ANOVA test with Dunnett's multiple comparison test. Error bars indicate SEM.

Figure S3

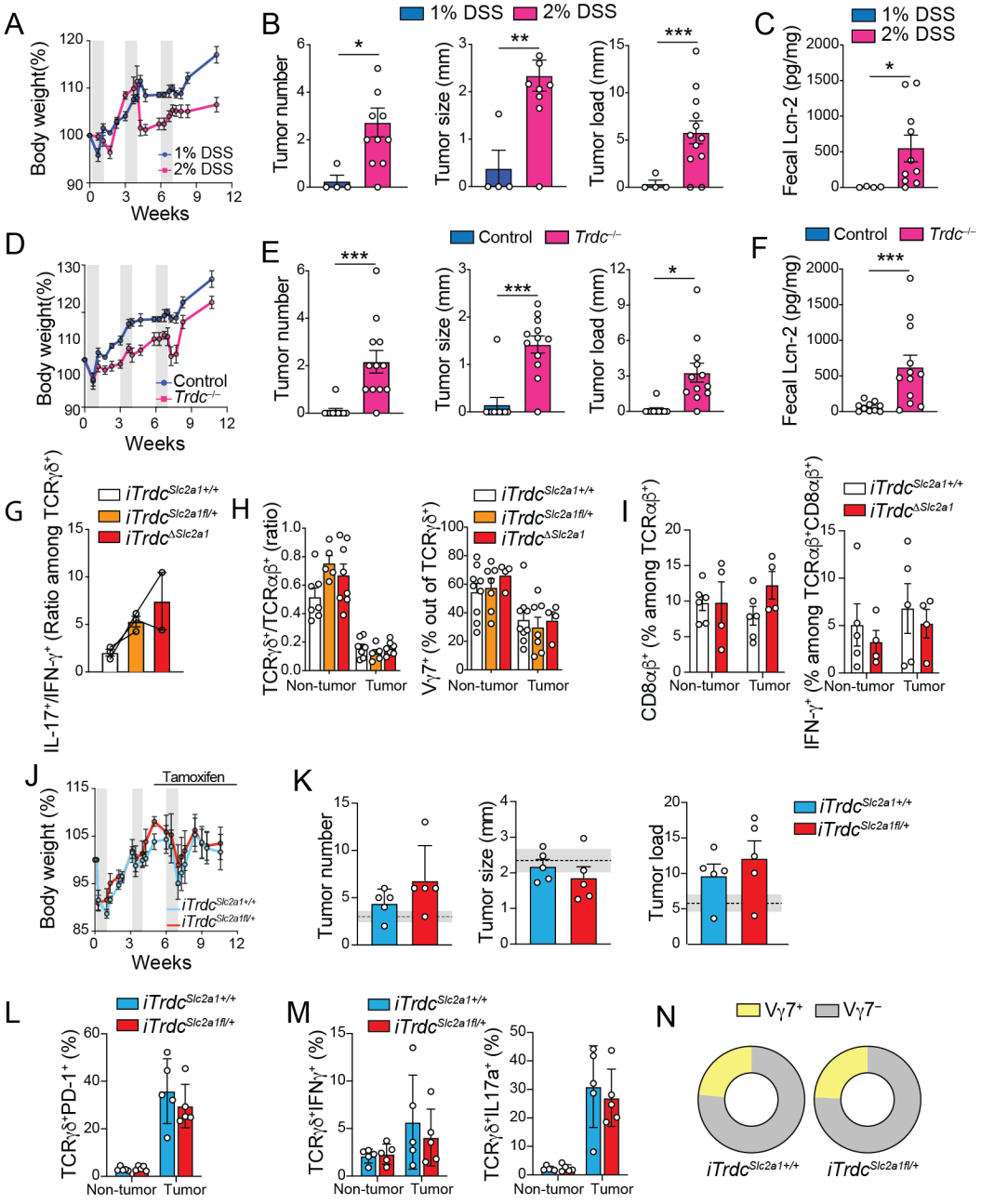
A
Body weight(%)
Weeks
1% DSS
2% DSS
B
1% DSS
2% DSS
Tumor number
Tumor size (mm)
Tumor load (mm)
C
Fecal Lcn-2 (pg/mg)
D
Body weight(%)
Weeks
Control
Trdc−/−
E
Control
Trdc−/−
Tumor number
Tumor size (mm)
Tumor load (mm)
F
Fecal Lcn-2 (pg/mg)
G
IL-17+/IFN-γ+ (Ratio among TCRγδ+)
iTrdcSlc2a1+/+
iTrdcSlc2a1fl/+
iTrdcΔSlc2a1
H
TCRγδ+/TCRαβ+ (ratio)
Vγ7+ (% out of TCRγδ+)
Non-tumor
Tumor
I
CD8αβ+ (% among TCRαβ+)
IFN-γ+ (% among TCRαβ+CD8αβ+)
J
Tamoxifen
Body weight (%)
Weeks
K
Tumor number
Tumor size (mm)
Tumor load
L
TCRγδ+PD-1+ (%)
M
TCRγδ+IFNγ+ (%)
TCRγδ+IL17a+ (%)
N
Vγ7+
Vγ7−

Figure S3. Supporting data to Figure 3. (**A-C**) C57BL/6 mice were injected with 12.5mg/kg of Azoxymethane (AOM) 2 days prior to three rounds of 1 week "on" and 2 weeks "off" of 1% vs 2% Dextran sodium sulfate (DSS) in the drinking water. (**A**) Mean percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (**B**) Tumor number, size, and load. (**C**) Fecal lipocalin-2 quantification. (**D-F**) $Trdc^{-/-}$ and C57BL/6 control mice were subjected to AOM-DSS model. (**D**) Mean percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (**E**) Tumor number, size, and load. Shaded area bounded by dashed lines indicates mean ± SEM of all control C57BL6/J mice analyzed in fig. S3B. (**F**) Fecal lipocalin-2 quantification. (**G-N**) $iTrdc^{\Delta Slc2a1}$, $iTrdc^{Slc2a1fl/+}$ and $iTrdc^{Slc2a1+/+}$ littermate control mice were subjected to AOM-DSS model, treated with tamoxifen for the first 3 weeks (**G-I**) or the last 6 weeks (**J-N**) and analyzed 12 weeks after initial AOM injection. (**G-I; L-N**) Flow cytometry analysis of colonic γδ T cells. (**G**) Ratio of IL-17^+ to IFN-γ^+ cells among tumor infiltrating TCR$\gamma\delta^+$ cells. (**H**) TCR$\gamma\delta^+$/TCR$\alpha\beta^+$ ratio among $CD45^+$ cells (right) and frequency of V $\gamma7^+$ among TCR $\gamma\delta^+$ cells. (**I**) Frequency of CD8$\alpha\beta^+$ T cells among TCR$\alpha\beta^+$ cells (left) and IFN-γ^+ among TCR$\alpha\beta^+$CD8$\alpha\beta^+$ cells (right). (**J**) Mean percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (**K**) Tumor number, size, and load. Shaded area bounded by dashed lines indicates mean ± SEM of all control C57BL6/J mice analyzed in fig. S3B (AOM+DSS model). (**L**) Frequency of PD-1^+ cells among TCR$\gamma\delta^+$ cells. (**M**) Frequency of IFN-γ^+ (left) and IL-17^+ (right) among TCR$\gamma\delta^+$ cells. (**N**) Frequency of Vγ usage by tumor infiltrating TCR$\gamma\delta^+$ cells. 1% vs 2% DSS data pooled from 2 experiments with 2-5 animals per group. $Trdc^{-/-}$ data pooled from 3 experiments with 3-5 animals per group. $iTrdc^{\Delta Slc2a1}$ representative data from 3 (early) and 2 (late) experiments with 3-5 animals per group. For cytokine staining, cells were stimulated with PMA and Ionomycin. *P < 0.05, **P < 0.01, ***P < 0.001. (**G-I, L-N**) One-way ANOVA test with Dunnett's multiple comparison test; (**B, C, E, F, K**) Two-tailed t-test; Error bars indicate SEM.

Figure S4

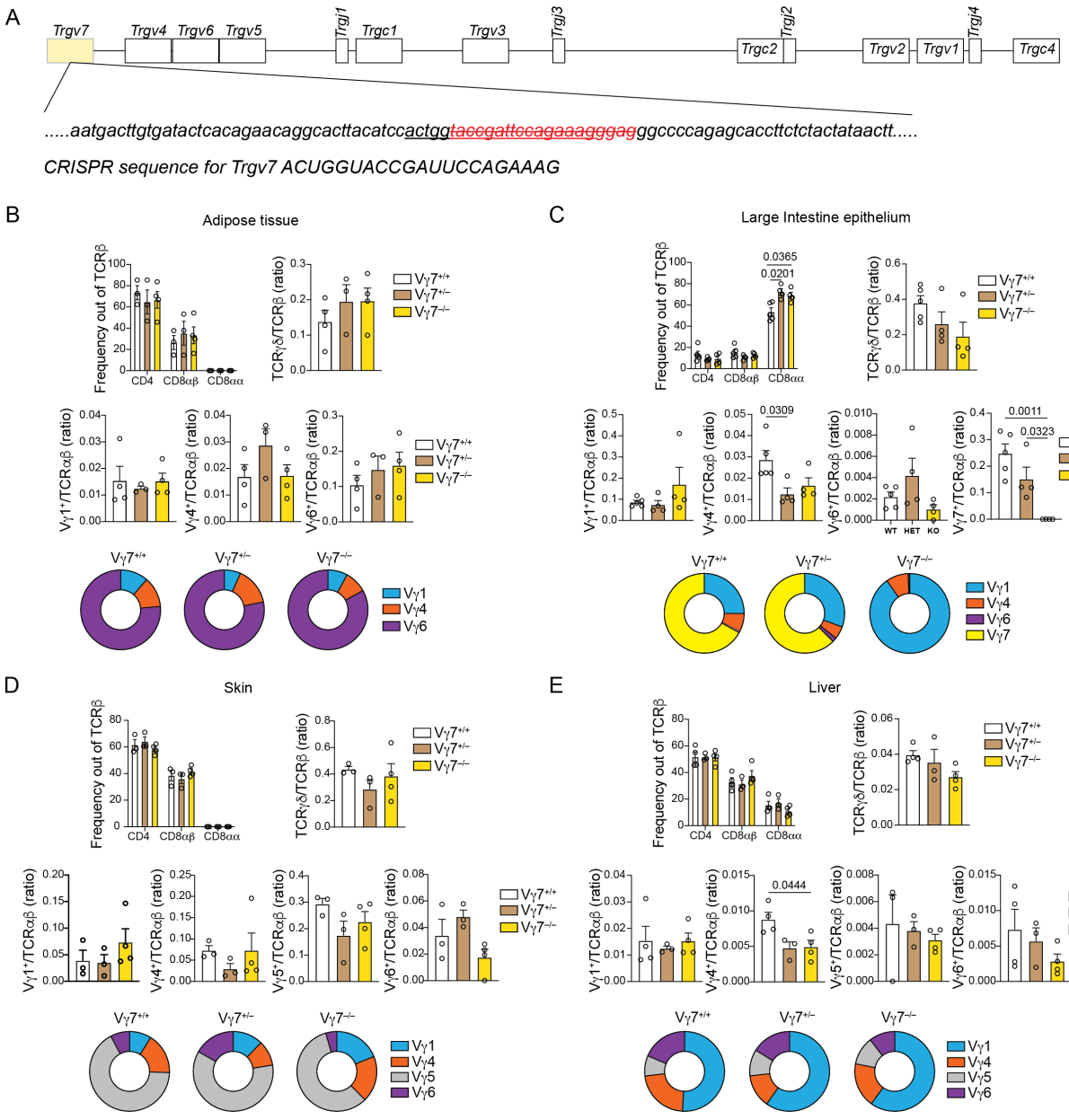
A
Trgv7
Trgv4
Trgv6
Trgv5
Trgj1
Trgc1
Trgv3
Trgj3
Trgc2
Trgj2
Trgv2
Trgv1
Trgj4
Trgc4
.....aatgacttgtgatactcacagaacaggcacttacatccactggtaccgattccagaaagggagggcccagagcaccttctctactataactt.....
CRISPR sequence for Trgv7 ACUGGUACCGAUUCCAGAAAG
B
Adipose tissue
C
Large Intestine epithelium
D
Skin
E
Liver
Frequency out of TCRβ
CD4
CD8αβ
CD8αα
TCRγδ/TCRβ (ratio)
Vγ7+/+
Vγ7+/−
Vγ7−/−
Vγ1+/TCRαβ (ratio)
Vγ4+/TCRαβ (ratio)
Vγ6+/TCRαβ (ratio)
Vγ7+/TCRαβ (ratio)
Vγ5+/TCRαβ (ratio)
0.0365
0.0201
0.0309
0.0011
0.0323
0.0444
WT
HET
KO
Vγ1
Vγ4
Vγ5
Vγ6
Vγ7

Figure S4. Supporting data to Figure 3. Characterization of Vγ7$^{-/-}$ mouse strain. (**A**) Generation of Vγ7 knockout mice by CRISPR deletion target. Schematic view of TCR gamma locus and CRISPR sequence used to partially delete *Trgv7* gene. Targeted sequence is shown underlined and deleted sequence is shown in red and strikethrough. (**B-E**) Flow cytometry analysis of αβ^{+} and γδ^{+} T cells from (**B**) adipose tissue, (**C**) large intestine epithelium (**D**) lung, and (**E**) liver tissue. Frequency of CD4^{+}, CD8αβ^{+} and C8αα^{+} among TCRαβ^{+} cells, and ratio of TCRγδ/αβ (top) and indicated Vγ/TCRαβ among CD45+ cells (center). Pie chart of frequency of Vγ usage among TCRγδ^{+} cells (bottom) from Vγ7$^{-/-}$, Vγ7$^{+/-}$ and Vγ7$^{+/+}$ littermate mice. Vγ7$^{-/-}$ strain was backcrossed to C57BL/6 for at least 10 generations. Data pooled from 2 experiments with 3 animals per group. *P < 0.05, **P < 0.01, ***P < 0.001. (**B-E**) One-way ANOVA test with Dunnett's multiple comparison test. Error bars indicate SEM.

Figure S5

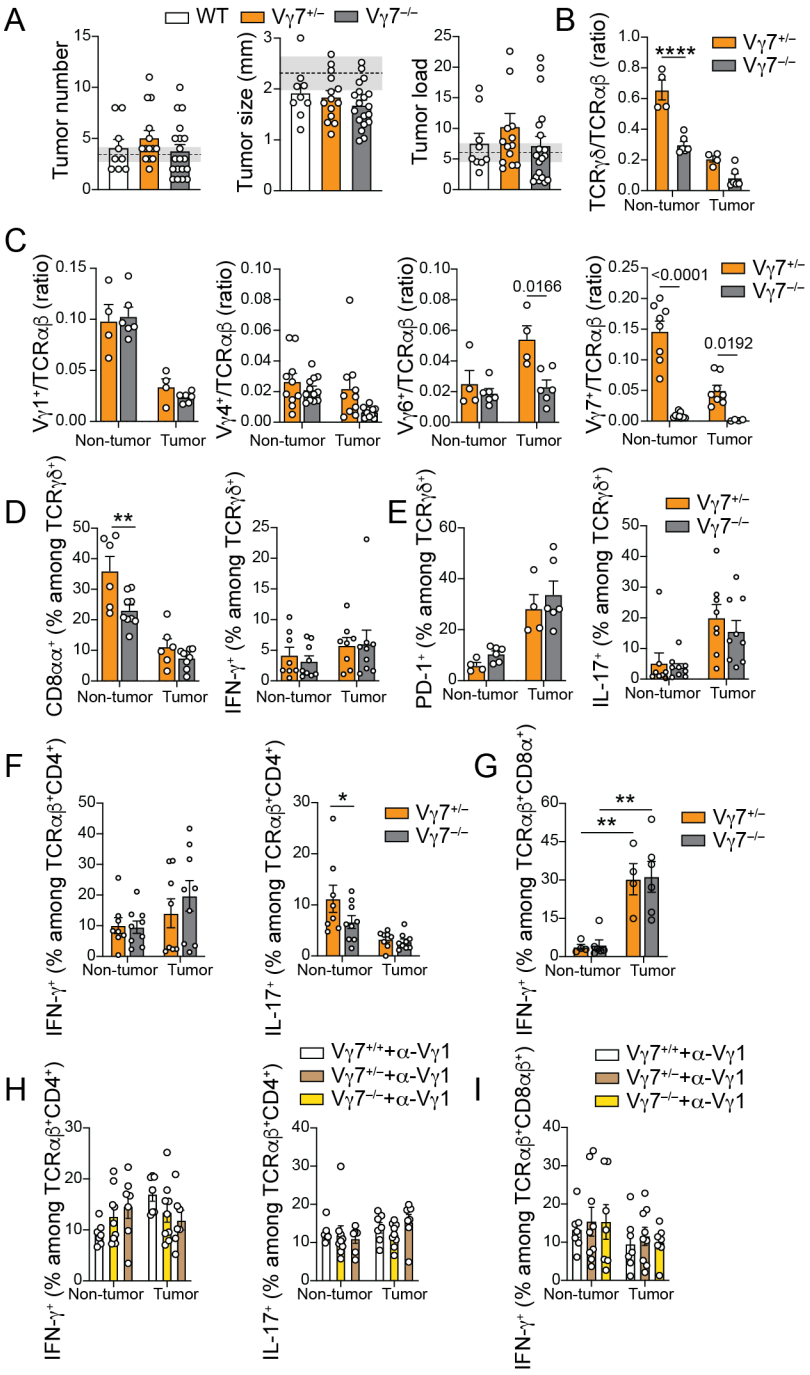
A
WT
Vγ7+/–
Vγ7–/–
Tumor number
Tumor size (mm)
Tumor load
B
TCRγδ/TCRαβ (ratio)
****
Non-tumor
Tumor
C
Vγ1+/TCRαβ (ratio)
Vγ4+/TCRαβ (ratio)
Vγ6+/TCRαβ (ratio)
Vγ7+/TCRαβ (ratio)
0.0166
<0.0001
0.0192
D
CD8αα+ (% among TCRγδ+)
**
IFN-γ+ (% among TCRγδ+)
E
PD-1+ (% among TCRγδ+)
IL-17+ (% among TCRγδ+)
F
IFN-γ+ (% among TCRαβ+CD4+)
IL-17+ (% among TCRαβ+CD4+)
*
G
IFN-γ+ (% among TCRαβ+CD8α+)
**
H
Vγ7+/+ +α-Vγ1
Vγ7+/– +α-Vγ1
Vγ7–/– +α-Vγ1
IFN-γ+ (% among TCRαβ+CD4+)
IL-17+ (% among TCRαβ+CD4+)
I
IFN-γ+ (% among TCRαβ+CD8αβ+)

Figure S5. Supporting data to Figure 3. (**A-I**) $V\gamma7^{-/-}$, $V\gamma7^{+/-}$ and $V\gamma7^{+/+}$ littermate control mice were subjected to AOM-DSS model. (**H, I**) All groups were treated twice a week with 200μg of anti-Vγ1 depleting antibody (2.11) 1 weeks prior AOM injection until the 2nd DSS treatment. (**A**) Tumor number, size, and load. Shaded area bounded by dashed lines indicates mean ± SEM of all control C57BL6/J mice analyzed in fig. S3B. (**B-I**) Flow cytometry analysis of γδ T cells from tumor or non-tumor colonic tissue. (**B**) TCRγδ/αβ ratio within $CD45^+$ cells. (**C**) Ratio of $V\gamma1^+$, $V\gamma4^+$, $V\gamma6^+$ and $V\gamma7^+$ cells to $TCR\alpha\beta^+$ cells. (**D**) Frequency of $CD8\alpha\alpha^+$ (left) and IFN-γ^+ (right) among $TCR\gamma\delta^+$ cells. (**E**) Frequency of PD-1^+ (left) and IL-17^+ (right) among $TCR\gamma\delta^+$ cells. (**F, H**) Frequency of IFN-γ^+ (left) and IL-17^+ (right) among $TCR\alpha\beta^+CD4^+$ cells. (**G, I**) Frequency of IFN-γ^+ among $TCR\alpha\beta^+CD8\alpha\beta^+$ cells. $V\gamma7^{-/-}$ data pooled from 3 (**A-G**) and 2 (**H, I**) experiments with 3-5 animals per group. For cytokine staining, cells were stimulated with PMA and Ionomycin. *P < 0.05, **P < 0.01, ***P < 0.001. (**A-I**) One-way ANOVA test with Dunnett's multiple comparison test; Error bars indicate SEM.

Figure S6

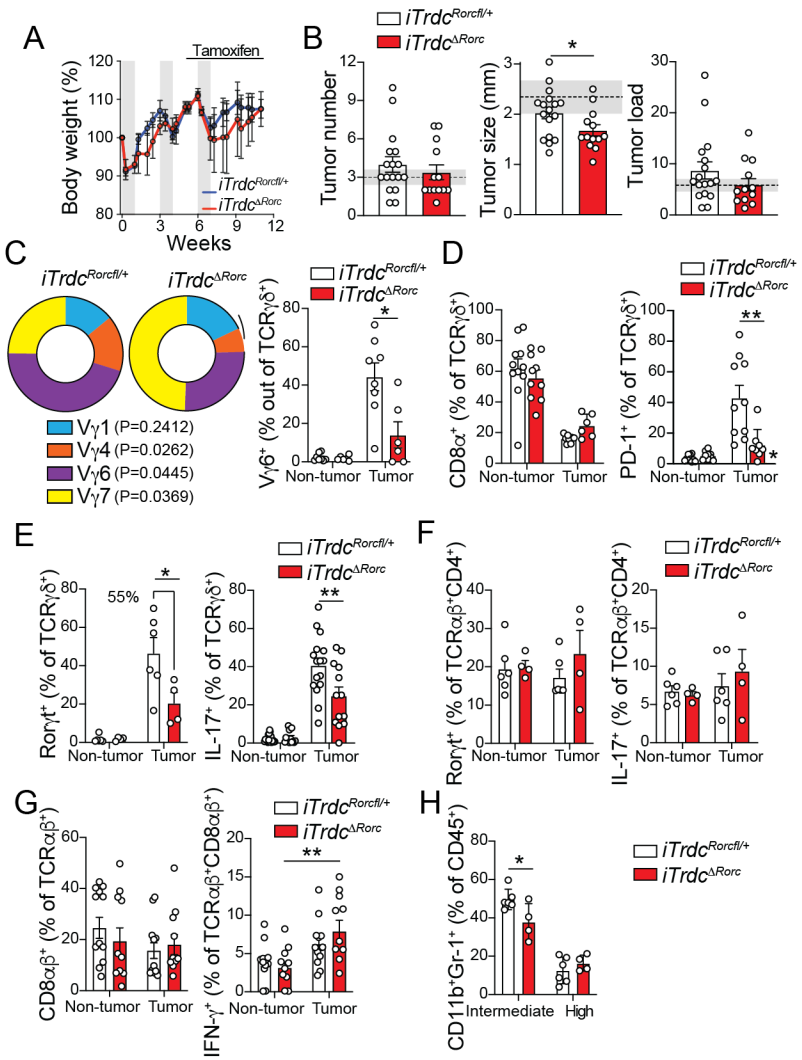
A
Tamoxifen
Body weight (%)
Weeks
iTrdc^Rorcfl/+
iTrdc^ΔRorc
B
Tumor number
Tumor size (mm)
Tumor load
C
Vγ1 (P=0.2412)
Vγ4 (P=0.0262)
Vγ6 (P=0.0445)
Vγ7 (P=0.0369)
Vγ6+ (% out of TCRγδ+)
Non-tumor
Tumor
D
CD8α+ (% of TCRγδ+)
PD-1+ (% of TCRγδ+)
E
Rorγt+ (% of TCRγδ+)
55%
IL-17+ (% of TCRγδ+)
F
Rorγt+ (% of TCRαβ+CD4+)
IL-17+ (% of TCRαβ+CD4+)
G
CD8αβ+ (% of TCRαβ+)
IFN-γ+ (% of TCRαβ+CD8αβ+)
H
CD11b+Gr-1+ (% of CD45+)
Intermediate
High

Figure S6. Supporting data to Figure 4. (**A-H**) iTrdc$^{\Delta Rorc}$ and iTrdc$^{Rorcfl/+}$ littermate control mice were subjected to AOM-DSS model, treated with tamoxifen for the last 6 weeks of the experiment and analyzed 12 weeks after initial AOM injection. (**A**) Mean percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (**B**) Tumor number, size, and load. Shaded area bounded by dashed lines indicates mean ± SEM of all control C57BL6/J mice analyzed in fig. S3B (AOM+DSS model). (**C-H**) Flow cytometry analysis of γδ T cells from tumor and non-tumor colonic tissue. (**C**) Frequency of Vγ usage (pie chart) and frequency of Vγ6$^+$ among TCRγδ$^+$ cells isolated from tumor. (**D**) Frequency of CD8α$^+$ (left) and PD-1$^+$ (right), and (**E**) Rorγt$^+$ (left) and IL-17$^+$ (right) cells among TCRγδ$^+$ cells. (**F**) Frequency of Rorγt$^+$ (left) and IL-17$^+$ (right) among TCRαβ$^+$CD4$^+$ cells. (**G**) Frequency of CD8αβ$^+$ among TCRαβ$^+$ cells (left) and IFN-γ$^+$ among TCRαβ$^+$CD8αβ$^+$ cells (right). (**H**) Frequency of tumor infiltrating CD11b$^+$Gr-1^{int} and CD11b$^+$Gr-1^{high} among CD45$^+$ cells. Data from iTrdc$^{\Delta Rorc}$ are pooled from 3 independent experiments with 3-6 animals per group. Experimental and control mice were co-housed. (**C-H**) One-way ANOVA with Dunnett's multiple comparison test; (**B, C**) two-tailed t-test. For cytokine staining, cells were stimulated with PMA and Ionomycin. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars indicate SEM.

Figure S7

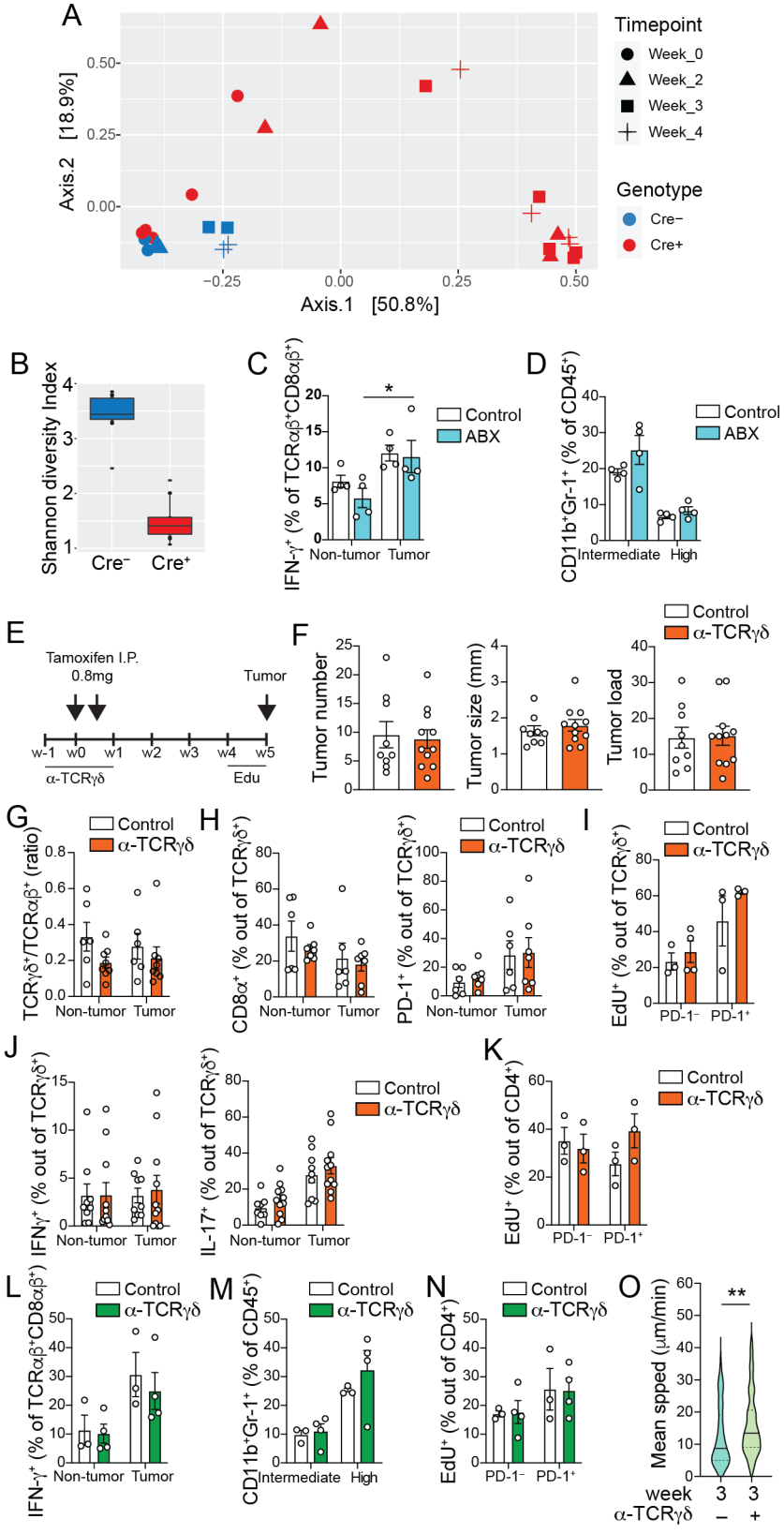
A
Timepoint
Week_0
Week_2
Week_3
Week_4
Genotype
Cre−
Cre+
Axis.2 [18.9%]
Axis.1 [50.8%]
B
Shannon diversity Index
Cre⁻
Cre⁺
C
IFN-γ⁺ (% of TCRαβ⁺CD8αβ⁺)
Control
ABX
Non-tumor
Tumor
D
CD11b⁺Gr-1⁺ (% of CD45⁺)
Intermediate
High
E
Tamoxifen I.P.
0.8mg
Tumor
w-1 w0 w1 w2 w3 w4 w5
α-TCRγδ
Edu
F
Tumor number
Tumor size (mm)
Tumor load
Control
α-TCRγδ
G
TCRγδ⁺/TCRαβ⁺ (ratio)
H
CD8α⁺ (% out of TCRγδ⁺)
PD-1⁺ (% out of TCRγδ⁺)
I
EdU⁺ (% out of TCRγδ⁺)
PD-1⁻
PD-1⁺
J
IFN-γ⁺ (% out of TCRγδ⁺)
IL-17⁺ (% out of TCRγδ⁺)
K
EdU⁺ (% out of CD4⁺)
L
IFN-γ⁺ (% of TCRαβ⁺CD8αβ⁺)
M
CD11b⁺Gr-1⁺ (% of CD45⁺)
N
EdU⁺ (% out of CD4⁺)
O
Mean spped (μm/min)
**
week
3 3
α-TCRγδ
– +

Figure S7. Supporting data to Figure 4. (**A, B**) Bacterial 16S ribosomal RNA amplicon (16S rRNA) sequencing from fecal pellets of tamoxifen-treated iCdx2$^{\Delta APC}$ and APC$^{fl/fl}$ littermate control mice. (**A**) Bi-dimensional principal coordinate analysis plot from iCdx2$^{\Delta APC}$ (Cre+ red) and APC$^{fl/fl}$ control mice (Cre– blue) before (week 0), and 2, 3 and 4 weeks after tamoxifen administration. (**B**) Shannon alpha diversity plot from iCdx2$^{\Delta APC}$ (Cre+ red) and APC$^{fl/fl}$ control mice (Cre– blue), four weeks after tamoxifen administration. (**C-O**) iCdx2$^{\Delta APC}$ mice were treated with 2 i.p. injections of 0.8mg tamoxifen and analyzed 5 weeks after. (**C, D**) Mice were treated with broad spectrum antibiotic (ABX) in the drinking water for the last 2 week of the experiment. (**C**) Frequency of IFN-γ^+ among TCR$\alpha\beta^+$CD8$\alpha\beta^+$ cells. (**D**) Frequency of tumor infiltrating CD11b$^+$Gr-1^{Int} and CD11b$^+$Gr-1^{high} among CD45$^+$ cells. (**E-K**) Mice were treated with 400μg of anti-TCRγδ blocking antibody (UC7-13D5) twice a week staring 1 week before until 1 week after the first tamoxifen injection, and EdU in the drinking water for the last week of the experiment. (**E**) Protocol. (**F**) Tumor number, size, and load. (**G-K**) Flow cytometry analysis of γδ T cells from tumor or non-tumor colonic tissue. (**G**) TCRγδ/αβ ratio within CD45$^+$ cells. (**H**) Frequency of CD8α$^+$ (left) and PD-1$^+$ (right) among TCRγδ$^+$ cells. (**I**) Frequency of EdU incorporation by PD-1$^-$ or PD-1$^+$ among TCRγδ$^+$ cells. (**J**) Frequency of IFN-γ^+ (left) and IL-17$^+$ (right) among TCRγδ$^+$ cells. (**K**) Frequency of EdU incorporation by PD-1$^-$ or PD-1$^+$ among TCR$\alpha\beta^+$CD4$^+$ cells. (**L-N**) Mice were treated with 400μg of anti-TCRγδ blocking antibody (UC7-13D5) twice a week for the last 2 weeks of the experiment, and EdU in the drinking water for the last week of the experiment. (**L**) Frequency of IFN-γ^+ among TCR$\alpha\beta^+$CD8$\alpha\beta^+$ cells. (**M**) Frequency of tumor infiltrating CD11b$^+$Gr-1^{Int} and CD11b$^+$Gr-1^{high} among CD45$^+$ cells. (**N**) Frequency of EdU incorporation by PD-1$^-$ or PD-1$^+$ among TCR$\alpha\beta^+$CD4$^+$ cells. (**O**) Intravital imaging of cecum γδ^{GFP} cells 3 weeks after tamoxifen injection. Mice were treated with 400μg of anti-TCRγδ blocking antibody (UC7-13D5) twice a week for the last week of the experiment. Cells were tracked by *Imaris* (Bitplane AG) software. Graph shows mean speed of tracks. Data from iCdx2$^{\Delta APC}$ antibiotic treated (ABX) are pooled from 3 experiments with 3-4 animals per group. 16S rRNA sequencing data representative of 2 experiments with 2-4 animals per group. Data from iCdx2$^{\Delta APC}$ antibody treated (UC7-13D5) are pooled from 2

experiments with 3-4 mice per group. Experimental and control mice were co-housed. (**C, G-N**) One-way ANOVA with Dunnett's multiple comparison test; (**D, F**) two-tailed t-test. For cytokine staining, cells were stimulated with PMA and Ionomycin. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars indicate SEM.

Figure S8

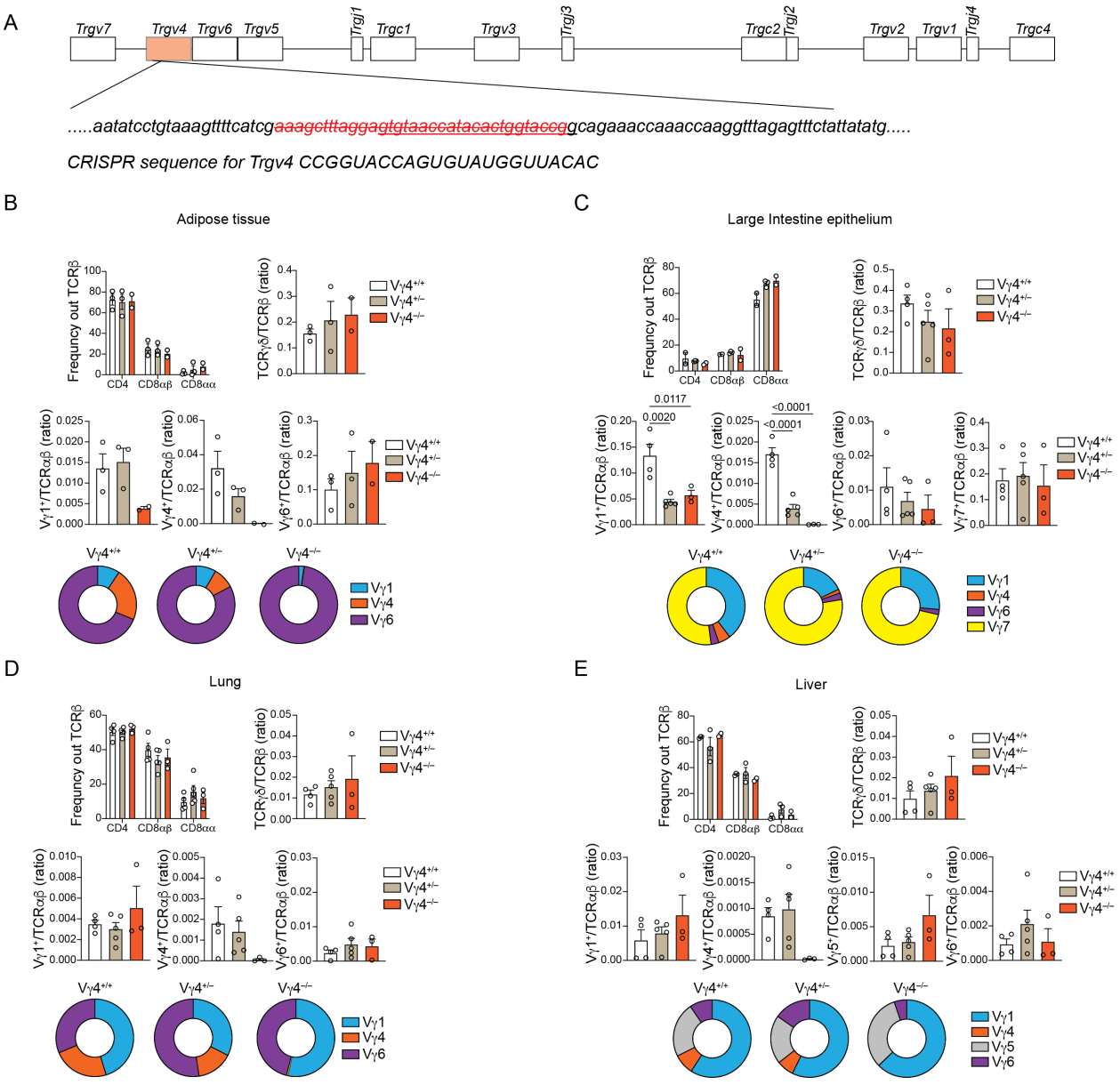
A
Trgv7
Trgv4
Trgv6
Trgv5
Trgj1
Trgc1
Trgv3
Trgj3
Trgc2
Trgj2
Trgv2
Trgv1
Trgj4
Trgc4
.....aatatcctgtaaagttttcatcgaaagctttaggagtgtaaccatacactggtaccggcagaaaccaaaccaaggtttagagtttctattatatg.....
CRISPR sequence for Trgv4 CCGGUACCAGUGUAUGGUUACAC
B
Adipose tissue
C
Large Intestine epithelium
D
Lung
E
Liver
Frequncy out TCRβ
TCRγδ/TCRβ (ratio)
Vγ1+/TCRαβ (ratio)
Vγ4+/TCRαβ (ratio)
Vγ6+/TCRαβ (ratio)
Vγ7+/TCRαβ (ratio)
Vγ5+/TCRαβ (ratio)
CD4
CD8αβ
CD8αα
Vγ4+/+
Vγ4+/−
Vγ4−/−
Vγ1
Vγ4
Vγ5
Vγ6
Vγ7
0.0117
0.0020
<0.0001
<0.0001

Figure S8. Supporting data to Figure 5. Characterization of Vγ4$^{-/-}$ mouse strain. (**A**) Generation of Vγ4 knockout mice by CRISPR deletion target. Schematic view of TCR gamma locus and CRISPR sequence used to partially delete *Trgv4* gene. Targeted sequence is shown underlined and deleted sequence is shown in red and strikethrough. (**B-E**) Flow cytometry analysis of αβ^{+} and γδ^{+} T cells from (**B**) adipose tissue, (**C**) large intestine epithelium (**D**) lung, and (**E**) liver. Frequency of CD4^{+}, CD8αβ^{+} and C8αα^{+} among TCRαβ^{+} cells, and ratio of TCRγδ/αβ (top) and indicated Vγ/TCRαβ among CD45+ cells (center). Pie chart of frequency of Vγ usage among TCRγδ^{+} cells (bottom) from Vγ4$^{-/-}$, Vγ4$^{+/-}$ and Vγ4$^{+/+}$ littermate mice. Vγ4$^{-/-}$ strain was backcrossed to C57BL/6 mice for at least 10 generations. Data pooled from 2 experiments with 3 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001. (**B-E**) One-way ANOVA test with Dunnett's multiple comparison test. Error bars indicate SEM.

Figure S9

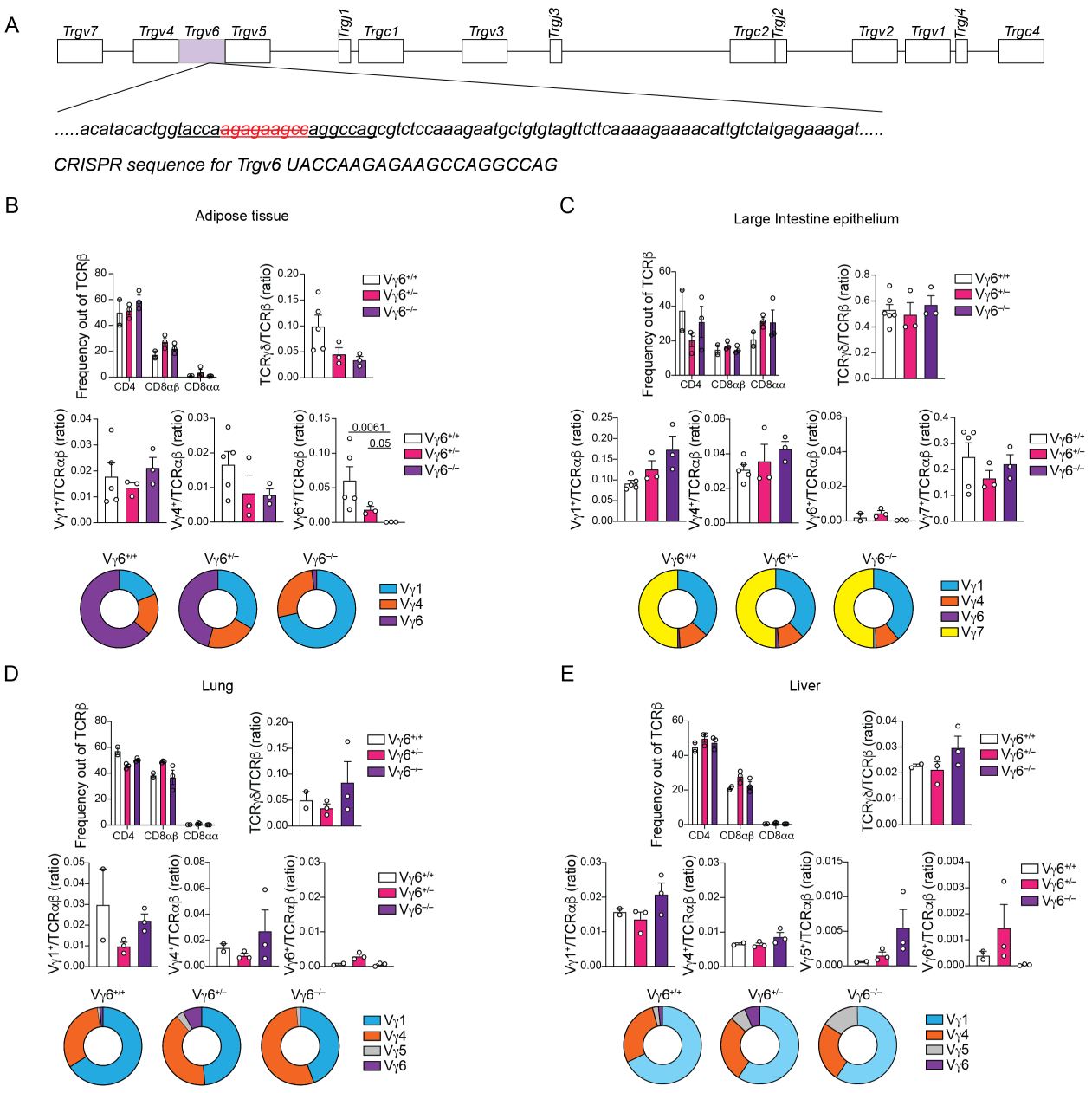
A
Trgv7
Trgv4
Trgv6
Trgv5
Trgj1
Trgc1
Trgv3
Trgj3
Trgc2
Trgj2
Trgv2
Trgv1
Trgj4
Trgc4
.....acatacactggtaccaagaggaagccaggccagcgtctccaaagaatgctgtgtagttcttcaaaagaaaacattgtctatgagaaagat.....
CRISPR sequence for Trgv6 UACCAAGAGAAGCCAGGCCAG
B
Adipose tissue
C
Large Intestine epithelium
D
Lung
E
Liver
Frequency out of TCRβ
TCRγδ/TCRβ (ratio)
Vγ1+/TCRαβ (ratio)
Vγ4+/TCRαβ (ratio)
Vγ5+/TCRαβ (ratio)
Vγ6+/TCRαβ (ratio)
Vγ7+/TCRαβ (ratio)
CD4
CD8αβ
CD8αα
Vγ6+/+
Vγ6+/−
Vγ6−/−
0.0061
0.05
Vγ1
Vγ4
Vγ5
Vγ6
Vγ7

Figure S9. Supporting data to Figure 5. Characterization of Vγ6$^{-/-}$ mouse strain. (**A**) Generation of Vγ6 knockout mice by CRISPR deletion target. Schematic view of TCR gamma locus and CRISPR sequence used to partially delete *Trgv6* gene. Targeted sequence is shown underlined and deleted sequence is shown in red and strikethrough. (**B-E**) Flow cytometry analysis of αβ^{+} and γδ^{+} T cells from (**B**) adipose tissue, (**C**) large intestine epithelium (**D**) lung, and (**E**) liver. Frequency of CD4^{+}, CD8αβ^{+} and C8αα^{+} among TCRαβ^{+} cells, and ratio of TCRγδ/αβ (top) and indicated Vγ/TCRαβ among CD45+ cells (center). Pie chart of frequency of Vγ usage among TCRγδ^{+} cells (bottom) from Vγ6$^{-/-}$, Vγ6$^{+/-}$ and Vγ6$^{+/+}$ littermate mice. Vγ6$^{-/-}$ strain was backcrossed to C57BL/6 for at least 10 generations. Data pooled from 2 experiments with 3 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001. (**B-E**) One-way ANOVA test with Dunnett's multiple comparison test. Error bars indicate SEM.

Figure S10

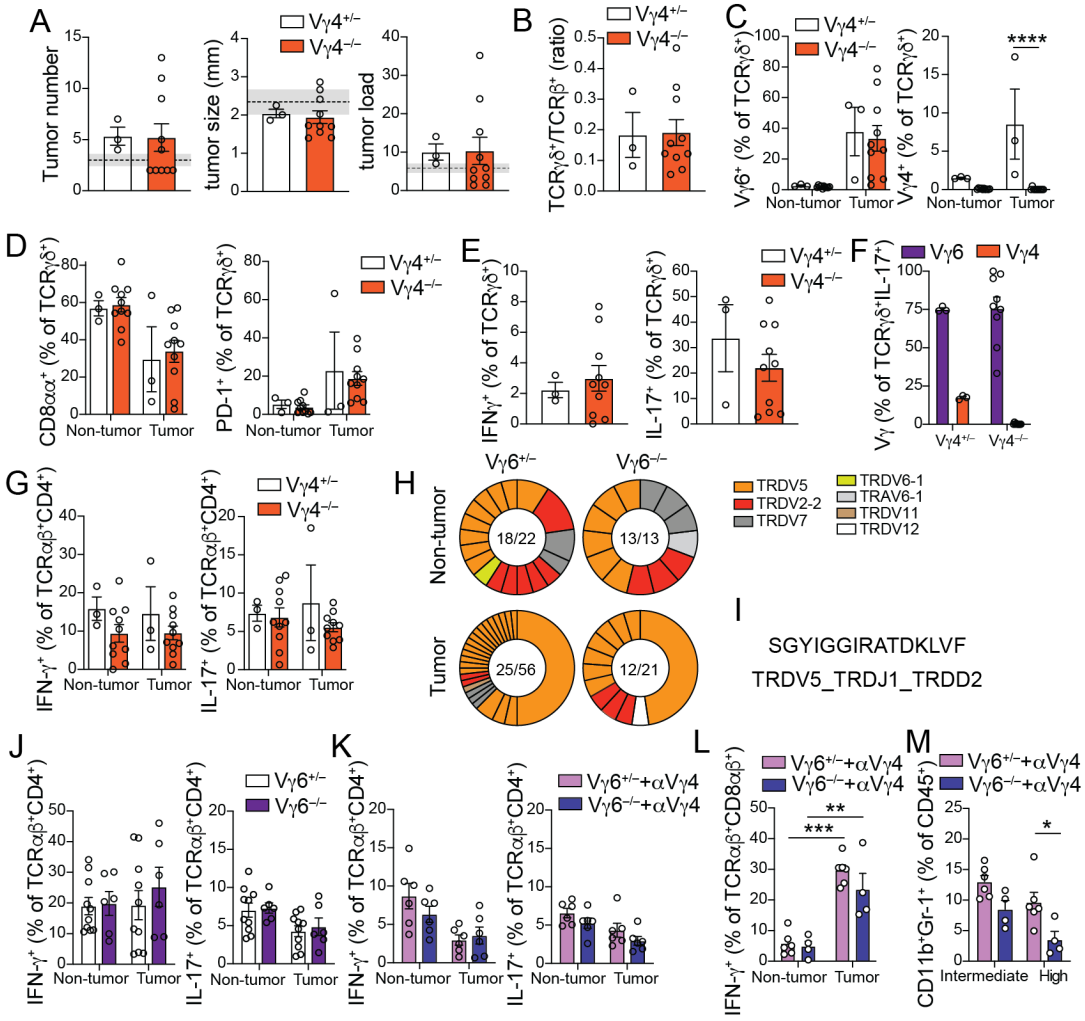
A
Tumor number
tumor size (mm)
tumor load
Vγ4+/–
Vγ4–/–
B
TCRγδ+/TCRβ+ (ratio)
C
Vγ6+ (% of TCRγδ+)
Vγ4+ (% of TCRγδ+)
****
Non-tumor
Tumor
D
CD8αα+ (% of TCRγδ+)
PD-1+ (% of TCRγδ+)
E
IFNγ+ (% of TCRγδ+)
IL-17+ (% of TCRγδ+)
F
Vγ6
Vγ4
Vγ+ (% of TCRγδ+IL-17+)
G
IFN-γ+ (% of TCRαβ+CD4+)
IL-17+ (% of TCRαβ+CD4+)
H
Vγ6+/–
Vγ6–/–
Non-tumor
Tumor
18/22
13/13
25/56
12/21
TRDV5
TRDV2-2
TRDV7
TRDV6-1
TRAV6-1
TRDV11
TRDV12
I
SGYIGGIRATDKLVF
TRDV5_TRDJ1_TRDD2
J
Vγ6+/–
Vγ6–/–
K
Vγ6+/–+αVγ4
Vγ6–/–+αVγ4
L
**
***
M
*
CD11b+Gr-1+ (% of CD45+)
Intermediate
High

Figure S10. Supporting data to Figure 5. (**A-G**) Female Vγ4$^{-/-}$ and Vγ4$^{+/-}$ littermate control mice, (**H-M**) Vγ6$^{-/-}$ and Vγ6$^{+/-}$ littermate control mice were subjected to the AOM-DSS model and analyzed 12 weeks after initial AOM injection. In panels **L** to **M**, mice received injections of 200μg of anti-Vγ4 depleting antibody (UC3-10A6) twice a week starting one week after the 2nd DSS cycle (last six weeks of experiment). (**A**) Tumor number, size, and load. Shaded area bounded by dashed lines indicates mean ± SEM of all control C57BL6/J mice analyzed in fig. S3B (AOM+DSS model). (**B-G**) Flow cytometry analysis of γδ T cells from tumor or non-tumor colonic tissue. (**B**) TCRγδ/αβ ratio within CD45^{+} cells from tumor colonic tissue. (**C**) Frequency of Vγ6^{+} (left) and Vγ4^{+} (right), and (**D**) CD8α^{+} (left) and PD-1^{+} (right) among TCRγδ^{+} cells. (**E**) Frequency of IFN-γ^{+} (left) and IL-17^{+} (right) among TCRγδ^{+} cells from tumor colonic tissue. (**F**) Frequency of Vγ6^{+} and Vγ4^{+} among IL-17–producing γδ T cells in tumor colonic tissue. (**G**) Frequency of IFN-γ $^{+}$ (left) and IL-17^{+} (right) among TCRαβ^{+}CD4^{+} cells. (**H**) Single-cell TCR sequencing of Vγ4^{+} cells (CD45^{+}TCRαβ^{-}TCRγδ^{+}) from tumor and non-tumor colonic tissue. Clones are colored based on Vδ usage. Orange clones represent expanded Vγ4Vδ5 cells. (**I**) Amino acid sequence of Vδ5 chain from expanded Vγ4Vδ5 clone. (**J, K**) Frequency of IFN-γ^{+} (left) and IL-17^{+} (right) among TCRαβ^{+}CD4^{+} cells. (**L**) Frequency of IFN-γ^{+} among TCRαβ^{+}CD8αβ^{+} cells. (**K**) Frequency of tumor infiltrating CD11b^{+}Gr-1^{Int} and CD11b^{+}Gr-1^{high} among CD45^{+} cells. Vγ4$^{-/-}$ data pooled from 2 experiments with 3-5 mice per group. For cytokine staining, cells were stimulated with PMA and Ionomycin. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (**C, D, F, G, J-M**) One-way ANOVA test with Dunnett's multiple comparison test; (**A, B, E**) two-tailed t-test. Error bars indicate SEM.