Dysfunctional tumor-infiltrating Vδ1+ T lymphocytes in microsatellite-stable colorectal cancer

Victoria Stary[1,2][*], Ram V. Pandey[3], Julia List[1], Lisa Kleissl[3], Florian Deckert[4], Julijan Kabiljo[1], Johannes Laengle[1], Vasileios Gerakopoulos[1], Rudolf Oehler[1], Lukas Watzke[5], Matthias Farlik[3], Samuel W. Lukowski[6], Anne B. Vogt[6], Georg Stary[3,4], Hannes Stockinger[2], Michael Bergmann[1,#], Nina Pilat[1,7,8,#]

1) Medical University of Vienna, Department of General Surgery, Division of Visceral Surgery, Comprehensive Cancer Center, Vienna, Austria.

2) Medical University of Vienna, Center for Pathophysiology, Infectiology and Immunology, Institute for Hygiene and Applied Immunology.

3) Medical University of Vienna, Department of Dermatology, Vienna, Austria.

4) CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria.

5) Medical University of Vienna, Department of Pathology, Vienna, Austria.

6) Department of Human Cancer Immunology, Boehringer Ingelheim RCV GmBH & Co KG., Dr. Boehringer Gasse 5-11, 1120 Vienna, Austria.

7) Medical University of Vienna, Department of Cardiac Surgery, Vienna, Austria.

8) Medical University of Vienna, Center for Biomedical Research and Translational Surgery, Vienna, Austria.

* Correspondence: Victoria Stary
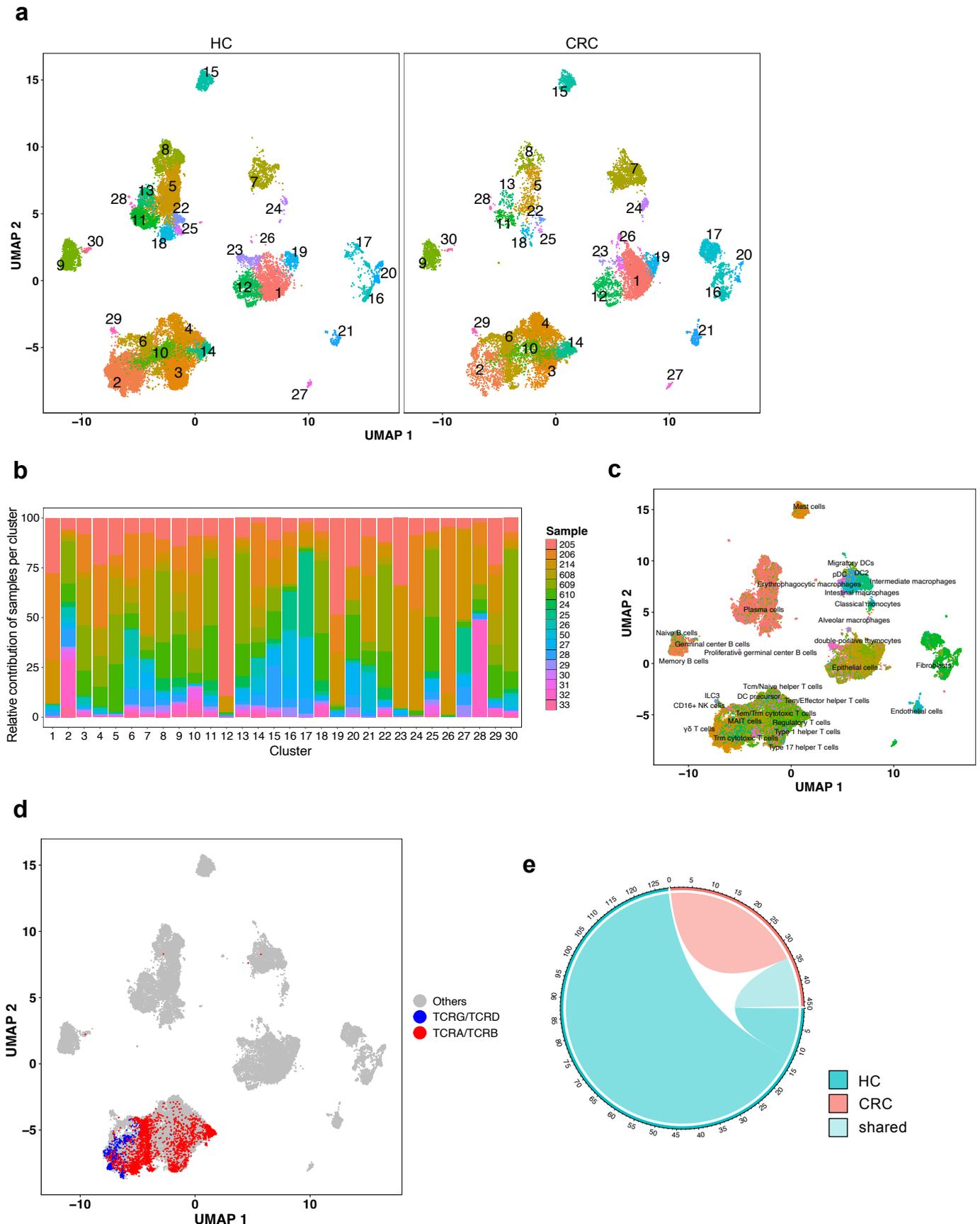
Figure S1.
(a) UMAP of cells in HC and CRC displayed according to the similarity of their transcriptome, resulting in 31 different color-coded clusters and cell identities. **(b)** Relative contribution of individual samples to each depicted cell cluster in HC and CRC. **(c)** Automated cell annotation using CellTypist on UMAP of HC and CRC displayed according to cell clusters. Mislabeled NK cells in fibroblast cluster were corrected using known marker genes for these cell subsets. **(d)** Overlay of clusters with TCR αβ- and γδ-repertoire. Cells with detectable αβ- and γδ-TCRs are colored in red and blue, respectively. **(e)** Circos plot demonstrating unique and shared clonotypes (light cyan) between HC (cyan) and CRC (pink).
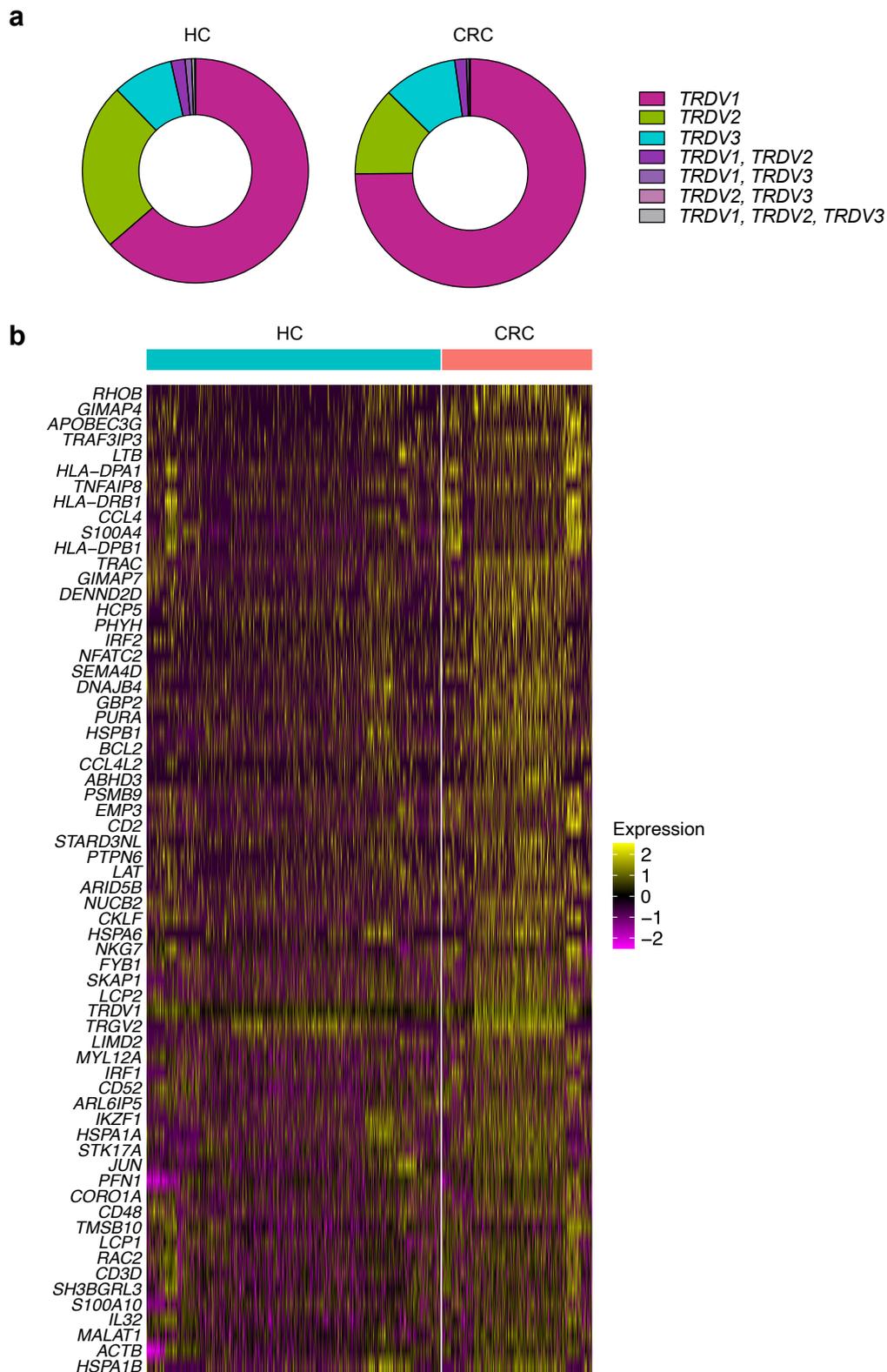
Figure S2.
**(a)** Donut charts of numbers of cells expressing *TRDV1, TRDV2, TRDV3,* doublets and triplets in HC and CRC. **(b)** Heatmap of differentially overexpressed genes in *TRDV1+* cells in HC compared to CRC.
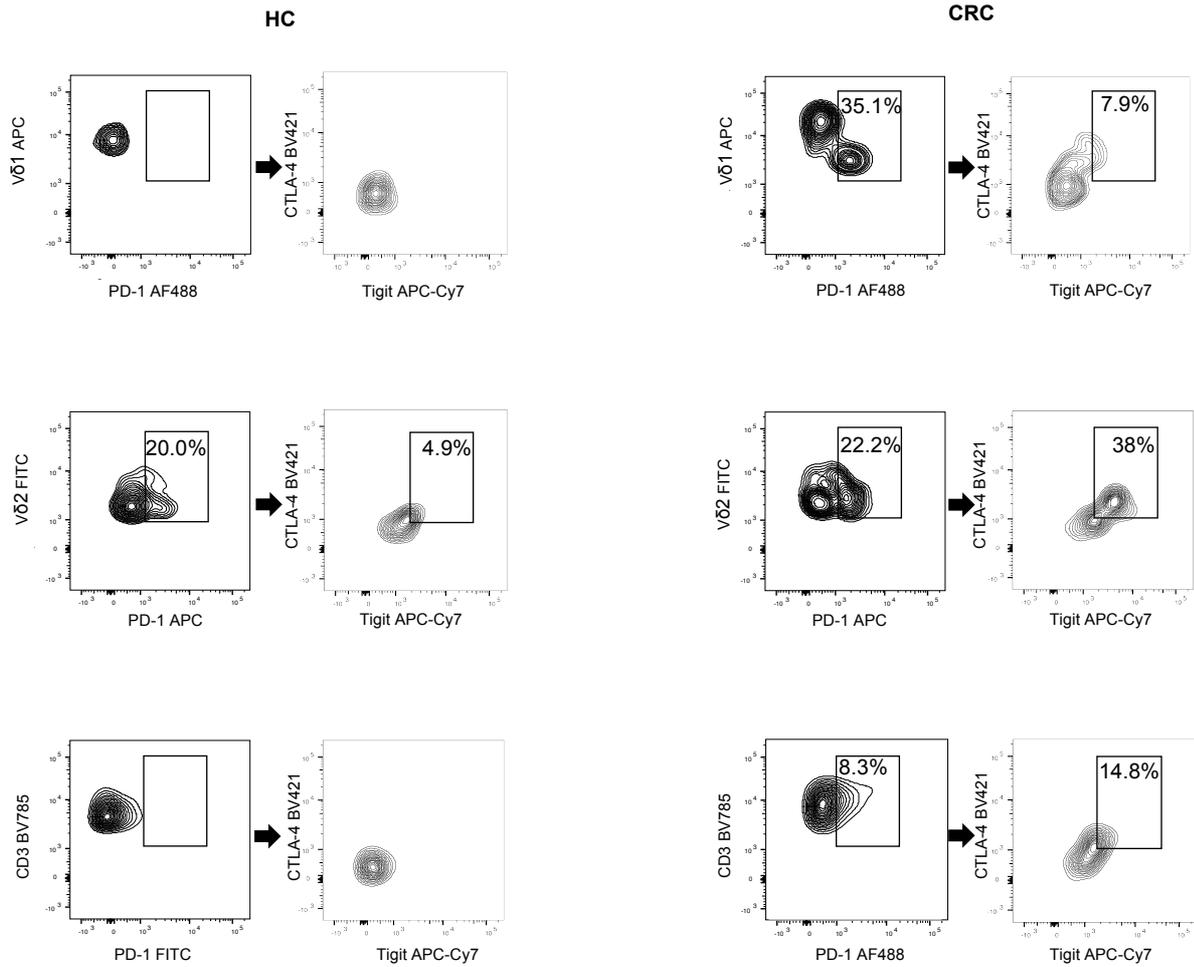
Figure S3.
Representative gating strategy of co-expression of CTLA-4 and TIGIT, first gated on PD-1, of Vδ1+, Vδ2+ and TCRγδ negative T cells in HC compared to CRC for Fig. 3h/3i.
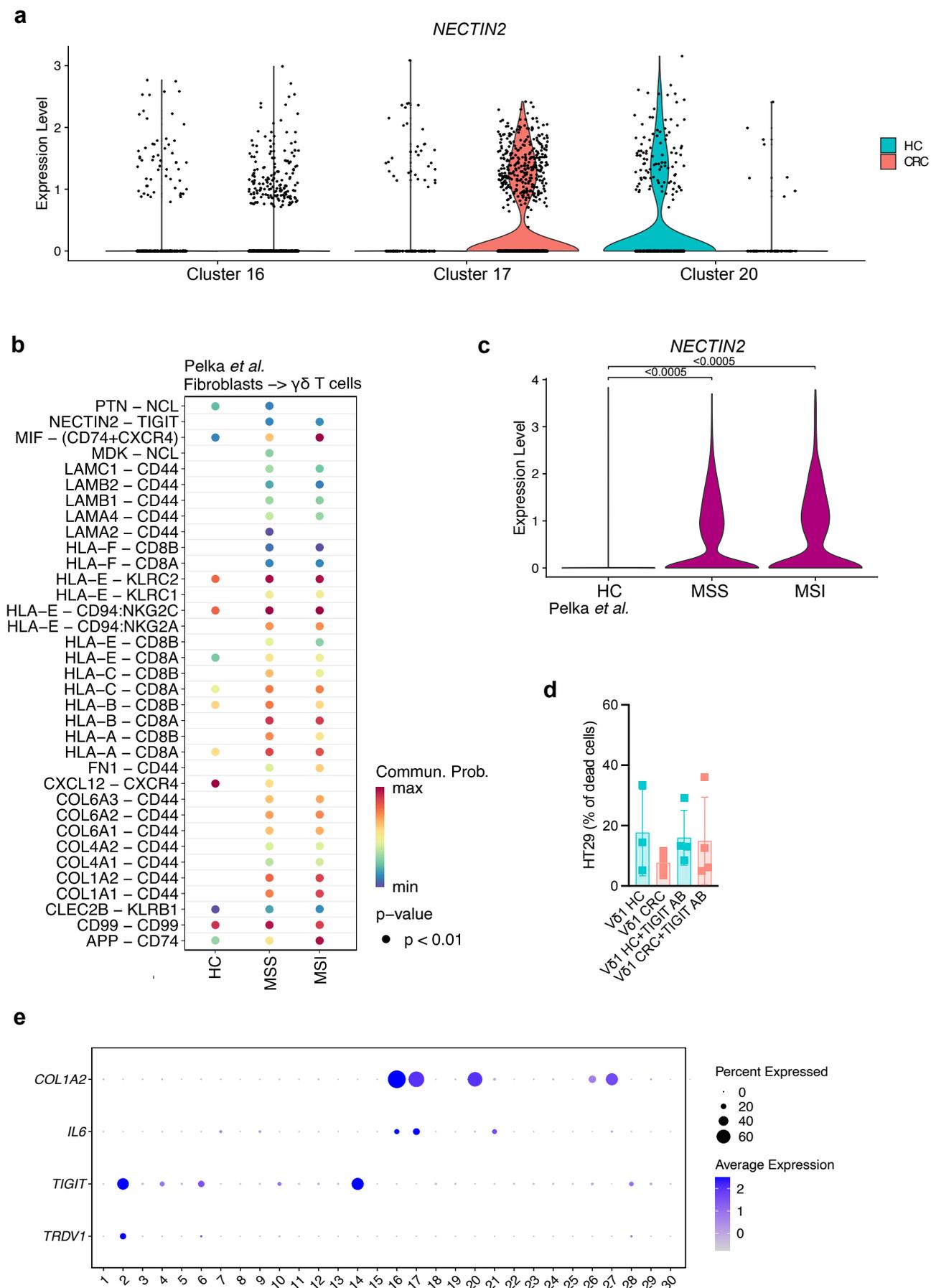
Figure S4.
(**a**) Violin plots depicting the expression of fibroblasts cluster 16, 17 and 20 between HC and CRC. (**b**) Significant receptor-ligand interactions of fibroblasts with γδ T cells in HC, MSS CRC and MSI CRC using the Pelka *et al.* data set[20]. (**c**) *Nectin2* expression of fibroblasts cluster in the Pelka *et al.* data set. (**d**) Control experiment displaying the percentage of dead HT29 cells after exclusion of baseline HT29 cell death in a killing experiment against Vδ1+ HC and Vδ1+ CRC without and with added TIGIT blocking antibody (except fibroblasts, n = 4). (**e**) Dot plot of average expression of depicted genes per cell cluster.
Data points and error bars represent the mean ± SD (**d**); Wilcoxon Rank Sum test (**b**), one-way ANOVA with Fisher's LSD (**c**).

Table S1: Characteristics of included patients

Sample ID	sex	age	histology	grading	T	N	amount N	M	primary tumor size in cm	localisation	sample preparation	enrichment	αβ-, γδ-TCR sequencing	HC	CRC	specifics
Sample1	f	54	AC	3	3	0	0	0	4	sigmoid colon						
Sample2	f	35	AC	3	3	0	0	0	6.7	sigmoid colon						
Sample3	f	50	AC	2	3	0	0	0	4	rectum						
Sample4	m	44	AC	2	2	1a	1	0	4	sigmoid colon						
Sample5	f	64	AC	2	2	0	0	0	6	rectum						
Sample6	f	72	AC	2	2	0	0	0	8	ascending colon						
Sample7	f	64	AC	2	3	0	0	0	6	ascending colon						
Sample8	m	60	AC	2	3	1b	2	0	4	sigmoid colon						
Sample9	m	87	AC	2	3	1a	1	0	4.5	ascending colon						
Sample10	f	57	AC	2	2	0	0	0	6	ascending colon						
Sample11	m	68	AC	2	3	2b	11	0	3.5	sigmoid colon						
Sample12	m	65	AC	2	3	0	0	0	4	sigmoid colon						
Sample13	m	51	AC	2	3	1b	2	1b	4	sigmoid colon						
Sample14	f	92	AC	2	2	0	0	0	4	rectum						
Sample15	f	42	AC	2	1	1a	1	1c	4	rectum						
Sample16	f	80	AC	2	3	0	0	0	10	ascending colon						
Sample17	m	57	AC	2	4b	1b	3	1c	12.5	sigmoid colon						
Sample18	f	63	AC	2	3	2a	4	1a	5	sigmoid colon						
Sample19	m	75	AC	2	4b	0	0	0	3.5	transverse colon						
Sample20	m	66	AC	3	3	0	0	0	0.5	sigmoid colon						
Sample21	m	70	AC	2	3	1b	2	0	4	ascending colon						
Sample22	f	66	AC	2	3	0	0	0	4.5	ascending colon						
Sample23	m	58	AC	3	2	0	0	0	4	sigmoid colon						
Sample87	f	56	AC	3	3	2b	15	0	4	rectum						
Sample88	f	85	AC	2	3	1a	1	0	1.5	ascending colon						
Sample89	m	72	AC	2	3	2a	4	0	3	sigmoid colon						
Sample92	m	68	AC	3	3	1a	1	0	3	ascending colon						
Sample97	f	80	AC	3	3	0	0	0	8	ascending colon						
Sample98	m	85	AC	2	3	0	0	0	4	ascending colon						
Sample99	m	76	AC	3	3	0	0	0	2	ascending colon						
Sample100	f	79	AC	3	3	1c	1	0	4	rectum						
Sample101	m	67	AC	2	3	1b	2	0	3.5	rectum						
Sample108	f	62	AC	3	3	2b	10	0	4	rectum						
Sample109	m	31	AC	2	4a	0	0	0	4	ascending colon						
Sample110	f	59	AC	1	1	0	0	0	3.5	ascending colon						
Sample111	M	76	AC	2	4a	1a	1	0	4	sigmoid colon						
Sample24	m	58	AC	2	3	1b	1	0	10	sigmoid colon	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable CD45+/- cells	X	X	X	-
Sample25	f	75	AC	2	3	0	0	0	3.5	sigmoid colon	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable CD45+/- cells	X	X	X	-
Sample26	m	84	AC	3	3	1b	3	1c	12	sigmoid colon	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable CD45+/- cells	X	X	X	-
Sample27	m	58	AC	2	3	1b	1	0	10	sigmoid colon	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable CD45+/- cells	X	X	X	-
Sample28	m	86	AC	2	3	1a	1	1a	7	ascending colon	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable CD45+/- cells	X	X	X	-
Sample29	f	77	AC	3	3	2a	4	0	2.5	ascending colon	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable CD45+/- cells	X	X	X	-
Sample30	f	34	AC	2	3	2a	4	0	3	rectum	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable 50% TCRγδ+ CD3+ cells, 50% CD45+/- cells	X	X	X	-
Sample31	f	41	AC	2	3	1b	2	0	2.5	rectum	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable 50% TCRγδ+ CD3+ cells, 50% CD45+/- cells	-	X	X	-
Sample32	f	82	AC	2	3	2a	4	0	3	ascending colon	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable 50% TCRγδ+ CD3+ cells, 50% CD45+/- cells	-	X	X	-
Sample33	m	47	AC	2	3	1c	0	1a	6	sigmoid colon	collagenase IV (10 U/ml) + deoxyribonuclease I (10mg/ml), gentleMACS Octo dissociator, 37°C_h_TDK1	viable 50% TCRγδ+ CD3+ cells, 50% CD45+/- cells	-	X	X	-
Sample205	f	78	AC	2	3	1b	2	0	6	transverse colon	TDK (130-095-929, Miltenyi), gentleMACS Octo dissociator	viable CD45+/- cells	-	X	X	CITE-seq: Totalseq-C Human Universal Cocktail v1.0 (399905)
Sample206	m	57	AC	2	3	1b	1	0	4	sigmoid colon	TDK (130-095-929, Miltenyi), gentleMACS Octo dissociator	viable CD45+/- cells	-	X	X	CITE-seq: Totalseq-C Human Universal Cocktail v1.0 (399905)
Sample214	m	38	AC	2	3	1b	2	0	7	rectum	TDK (130-095-929, Miltenyi), gentleMACS Octo dissociator	viable CD45+/- cells	-	X	X	
Sample608	m	59	AC	2	3	2b	20	0	4	sigmoid colon	TDK (130-095-929, Miltenyi), gentleMACS Octo dissociator	viable CD45+/- cells	-	X	-	CITE-seq: Totalseq-C Human Universal Cocktail v1.0 (399905)
Sample609	f	69	AC	2	3	1b	2	0	2	sigmoid colon	TDK (130-095-929, Miltenyi), gentleMACS Octo dissociator	viable CD45+/- cells	-	X	-	CITE-seq: Totalseq-C Human Universal Cocktail v1.0 (399905)
Sample610	m	56	AC	2	2	0	0	0	3.5	ascending colon	TDK (130-095-929, Miltenyi), gentleMACS Octo dissociator	viable CD45+/- cells	-	X	-	CITE-seq: Totalseq-C Human Universal Cocktail v1.0 (399905)
Sample50	f	78	AC	2	3	1b	2	0	6	transverse colon	collagenase IV (1:5), 45 min 37°C	viable 60% CD45+EpCAM- 20% CD45- EpCAM+, 20% CD45-EpCAM-	X	-	X	CITE-seq: Totalseq-C Human Universal Cocktail v1.0 (399905), anti-human CD172a, CD366, Siglec10, CD206, CD258

Grey used for sequencing, AC; adenocarcinoma, MS; microsatellite, TDK; Tumor Dissociation Kit

Table S2: Numbers of αβ- and γδ-TCRs for each sample in HC and CRC.

Sample	TCRαβ	TCRγδ
Sample24 HC	61	95
Sample24 CRC	2	0
Sample25 CRC	19	1
Sample26 HC	407	46
Sample26 CRC	716	36
Sample27 HC	70	32
Sample27 CRC	75	1
Sample28 HC	249	142
Sample28 CRC	263	9
Sample29 HC	80	4
Sample29 CRC	14	3
Sample30 HC	130	77
Sample30 CRC	89	102

Table S3: Antibodies used in this study.

Antibody specificity	Fluorochrome	Clone	Dilution	Company	Ref. number	
CD3	APC	SK7	1/100	BD Bioscience	345767	https://www.bdbiosciences.com/en-at/products/reagents/flow-cytometry-reagents/clinical-diagnostics/single-color-antibodies-asr-ivd-ce-ivd/cd3-apc.345767
CD3	BV786	UCHT1	1/100	BD Bioscience	565491	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/bv786-mouse-anti-human-cd3.565491
CD27	BV421	M-T271	1/100	BD Bioscience	562513	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/bv421-mouse-anti-human-cd27.562513
CD31	APC-Cy7	WM59	1/50	BioLegend	303119	https://www.biolegend.com/de-de/products/apc-cyanine7-anti-human-cd31-antibody-6730
CD45	PE/CF594	HI30	1/400	BD Bioscience	562279	https://www.bdbiosciences.com/en-at/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-cf594-mouse-anti-human-cd45.562279
CD45RA	APC-Cy7	HI100	1/100	BioLegend	304127	https://www.biolegend.com/de-de/products/apc-cyanine7-anti-human-cd45ra-antibody-7056
CD90	PE-Cy7	5E10	1/100	BioLegend	328124	https://www.biolegend.com/de-de/products/pe-cyanine7-anti-human-cd90-thy1-antibody-8282
CD107a	PE	H4A3	1/40	BioLegend	328607	https://www.biolegend.com/de-de/products/pe-anti-human-cd107a-lamp-1-antibody-4967
CD160	PerCp-Cy5.5	BY55	1/200	BioLegend	341210	https://www.biolegend.com/de-de/products/percp-cyanine5-5-anti-human-cd160-antibody-9082
CD244	PerCp-Cy5.5	C1.7	1/200	BioLegend	329516	https://www.biolegend.com/de-de/products/percp-cyanine5-5-anti-human-cd244-2b4-antibody-7511
CTLA-4	BV421	BNI3	1/50	BioLegend	369605	https://www.biolegend.com/de-de/products/brilliant-violet-421-anti-human-cd152-ctla-4-antibody-13159
EpCAM	BV421	CO17-1A	1/50	BioLegend	369821	https://www.biolegend.com/de-de/products/brilliant-violet-421-anti-human-cd326-epcam-antibody-14320
FasL	PE-Vio770	NOK-1	1/100	Miltenyi Biotec	130-104-270	https://www.miltenyibiotec.com/US-en/products/cd178-antibody-anti-human-nok-1.html#conjugate=pe-vio-770:size=100-tests-in-200-ul
GranzymeB	BV421	GB11	1/75	BD Bioscience	563389	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/bv421-mouse-anti-human-granzyme-b.563389
IFNγ	V450	B27	1/75	BD Bioscience	560372	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/v450-mouse-anti-human-ifn.560372
Ki-67	PE-Cy7	20Raj1	1/100	BD Bioscience	561283	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-cy-7-mouse-anti-ki-67.561283
LAG-3	PE-Cy7	11G3C65	1/50	BioLegend	369310	https://www.biolegend.com/de-de/products/pe-cyanine7-anti-human-cd223-lag-3-antibody-13551
PD1	APC	EH12.2H7	1/100	BioLegend	329908	https://www.biolegend.com/de-de/products/apc-anti-human-cd279-pd-1-antibody-4413
PD1	FITC	EH12.2H7	1/100	BioLegend	329903	https://www.biolegend.com/de-de/products/fitc-anti-human-cd279-pd-1-antibody-4411
Perforin	PerCp-Cy5.5	δG9	1/75	BD Bioscience	563762	https://www.bdbiosciences.com/en-at/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/percp-cy-5-5-mouse-anti-human-perforin.563762
TCRγδ	PE	B1	1/150	BioLegend	331210	https://www.biolegend.com/de-de/products/pe-anti-human-tcr-gamma-delta-antibody-4553
TCR Vδ1	APC	REA173	1/150	Miltenyi Biotec	130-118-968	https://www.miltenyibiotec.com/US-en/products/tcr-vd1-antibody-anti-human-reafinity-rea173.html#conjugate=apc:size=100-tests-in-200-ul

Table S3: Antibodies used in this study.

Antibody specificity	Fluorochrome	Clone	Dilution	Company	Ref. number	
TCR Vδ2	FITC	B6	1/80	BD Bioscience	555738	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/fitc-mouse-anti-human-v-2-tcr.555738
TIGIT	APC-Cy7	A15153G	1/50	BioLegend	372707	https://www.biolegend.com/de-de/products/apc-fire-750-anti-human-tigit-vstm3-antibody-13740
TIGIT	Blocking	A15153A	1/5000	BioLegend	613703	https://www.biolegend.com/de-de/products/ultra-leaf-purified-anti-human-tigit-antibody-18510?GroupID=GROUP28
Tim3	APC-Cy7	F38-2EZ	1/50	BioLegend	345026	https://www.biolegend.com/de-de/products/apc-cyanine7-anti-human-cd366-tim-3-antibody-11928
TNF-α	PE-Cy7	MAb11	1/75	BD Bioscience	560678	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-cy-7-mouse-anti-human-tnf.560678
TRAIL	Biotin	RIK-2	1/100	BD Bioscience	550431	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/biotin-mouse-anti-human-cd253.550431