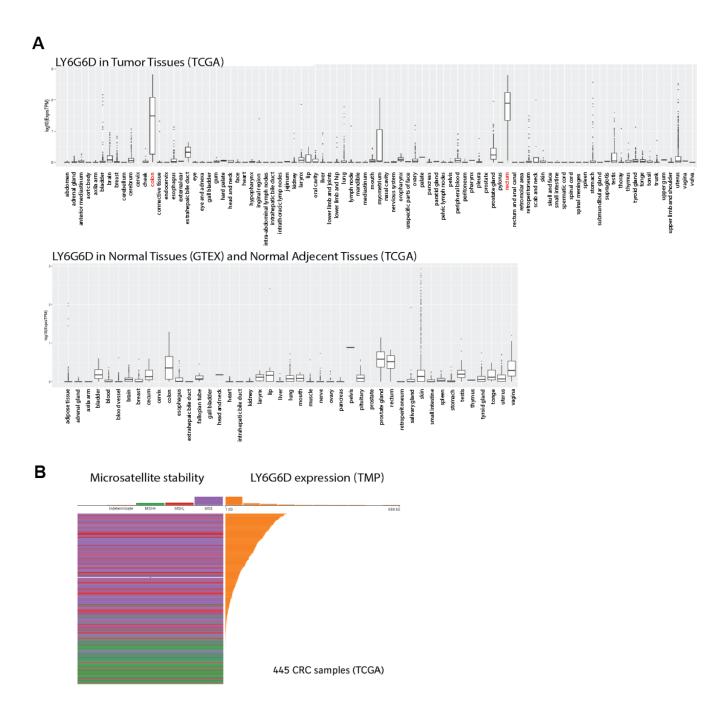
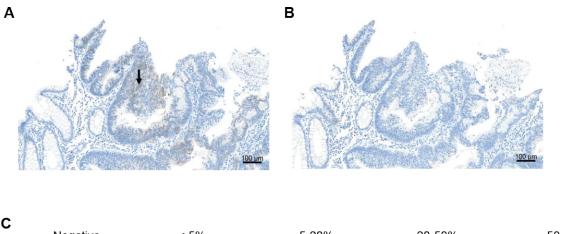
Supplemental Figure and Material and Methods

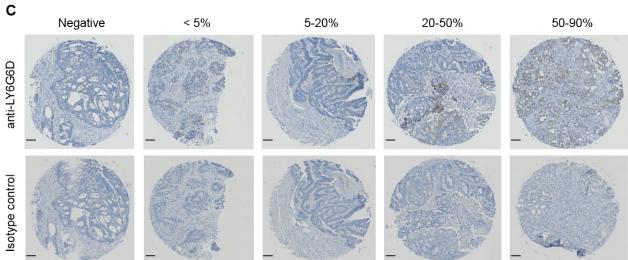
Supplemental Figure 1



Supplemental Figure 1.

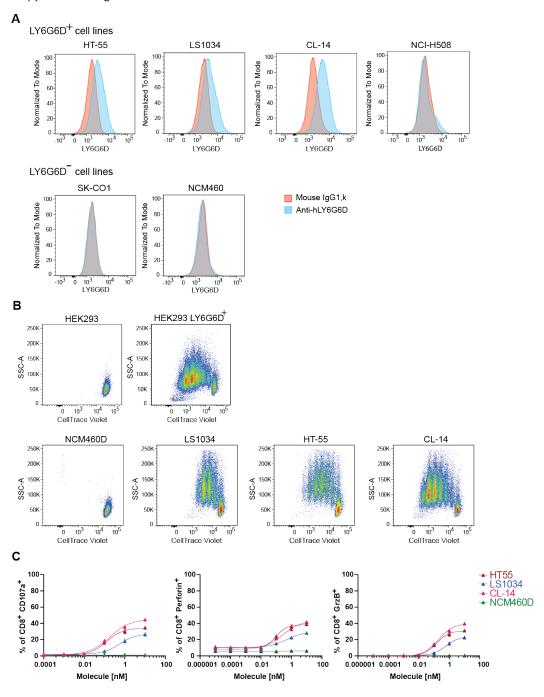
(A) LY6G6D mRNA expression in tumors (TCGA) and normal tissues (GTEX). (B) Correlation of LY6G6D mRNA expression and microsatellite instability status. MSI-H: microsatellite instability high (green); MSI-L: microsatellite instability low (red); MSS: microsatellite stable (purple).





Supplemental Figure 2.

(A-B) Antibody absorption test by LY6G6D recombinant protein pre-incubation using CRC tissue sample. Anti-LY6G6D staining of tumor cells was detected in CRC tissue without pre-incubation of clone 10C1 with recombinant protein (A). No staining was detected in CRC tissue after pre-incubation of clone 10C1 with 50X recombinant protein (B). (C) Representative images of CRC samples with different percentage of LY6G6D stained cells within the tumor area stained with clone clone 10C1 (top), or isotype control (bottom). Bar length is 100 μ m.



Supplemental Figure 3.

(A) LY6G6D expression on CRC cell lines. (B) LY6G6D-negative (HEK293 and NCM460) and positive (HEK LY6G6D+, LS1034, HT-55 and CL-14) were co-incubated with purified labelled T cells and LY6G6D/CD3 TcE for 5 days. Proliferation of T cells was assessed by dilution of cell tracer. (C) LY6G6D-negative and positive tumor cells were co-incubated with purified T cells and increasing amounts of concentrations of LY6G6D/CD3 TcE. Degranulation markers were analyzed by FACs after 72 hours of incubation.

Supplemental Figure 4 Α **Baseline expression** Tissue # Tumor type LY6G6D CD3 CRC-MSS positive positive 2 CLM positive positive 3 CRC-MSS positive negative positive positive 4 CLM-MSS 5 CLM-MSS positive positive 6 CRC-MSS positive positive CRC-MSS positive negative CRC-MSI H positive negative С В LY6G6D negative LY6G6D negative IHC: met CRC met CRC Granzyme B IHC: TNP/CD3 TcE EpCAM 48h treatment IHC: EpCAM/CD3 TcE CD3 48h treatment D 40000 IFNγ 150000 IP-10 Granzyme B Granzyme B [pg/mL] 10000-IP-10 [pg/mL] 30000 100000 20000 50000 10000 10000 THE CANCES TEN THE BRICHS TEE Tecepic Da Tek THE ICLD 3 TOE THRICO3 TEE ERCANCOSTEE THEICOSTER 0 ERCANCO3 TEE EpCAMICO3 TEE 600 IL-2 80 TNFα 150000 MCP-1 MCP-1 [pg/mL] TNFa [pg/mL] 60 IL-2 [pg/mL] 400 100000

40

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TEE BOOD TEE

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ERCANCOS TEL

Supplemental Figure 4.

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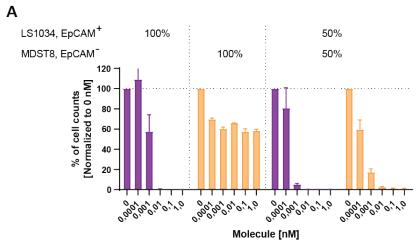
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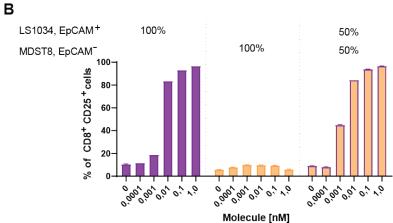
(A) Information on CRC phenotype and baseline expression of LY6G6D and CD3 in CRC tissue samples. (B) EpCAM and CD3 expression in one LY6G6D- CRC tissue sample (tissue #3) at baseline. (C-D) Tissue #3 was cultured for with 1 nM LY6G6D/CD3 TcE, 1nM EpCAM/CD3 TcE or control TNP/CD3 TcE. After 48 hours, Granzyme B staining in the tissue slides (C) and cytokines in the supernatant (D) were assessed.

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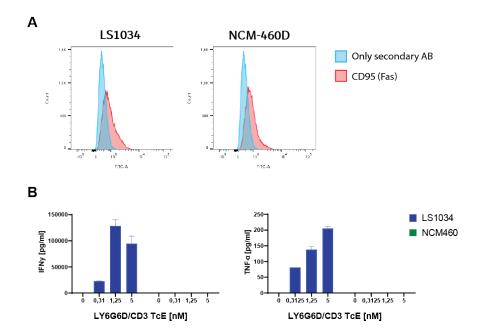
EDCAMCO STOR





Supplemental Figure 5.

(A-B) EpCAM-positive and -negative (LS1034 and MDST8, respectively) tumor cells were labeled with cell tracer, plated isolated (100%) or co-cultured at 1:1 ratio. Tumor cells were then co-incubated with purified T cells and increasing amounts of concentrations of EpCAM/CD3 TcE. The total number of tumor cells (A) and T cell activation (B) was analyzed after 72 hours of incubation.



Supplemental Figure 6.

(A) Basal expression of Fas (CD95) in LS1034 and NCM-460D cell lines. (B) LY6G6D-positive (LS1034) or negative (NCM460) tumor cells were co-incubated with purified T cells and increasing amounts of concentrations of LY6G6D/CD3 TcE. IFN γ and TNF α cytokines in the media were assessed after 72 hours of incubation

T cell Proliferation assay:

Target cells were seeded in 96-well plates and pre-incubated at 37°C for 3 hours. Purified T-cells from PBMC (EasySep™ Human T Cell Enrichment Kit; Stemcell Technologies), labelled with CellTrace™ Violet Dye according to manufacturer's instructions. And added in a target to T cell ratio 1:10, along with the LY6G6D/CD3 TcE. After 5 days of incubation T-cells were harvested and stained with Zombie NIR Fixable Viability Dye. T-cells were stained with anti-CD4 and antiCD8 (BioLegend; BD Biosciences) and acquired on FACS Canto-II (BD Biosciences). Analysis of proliferation was performed by FlowJo (TreeStar).

Table of reagents:

Reagent or Resource	Source	Identifier (Catalog #)
Antibodies		
Mouse anti-human CD45 (clone 2D1)	BioLegend	368509
Mouse anti-human CD8 (clone RPA-	BD Biosciences	562428
T8)		
Mouse anti-human CD4 (clone OKT4)	BioLegend	317444; 317414
Mouse anti-human CD25 (clone M-	BioLegend	356110
A251)		
Anti-human CD25 (clone BC96)	BioLegend	302606
Mouse anti-human CD69 (clone FN50)	BD Biosciences	557745
Human TruStain FcX	BioLegend	422302
Anti-human LAG3 (clone T47-530)	BD Biosciences	745640
Anti-human CTLA4 (clone BNI3)	BioLegend	369604
Anti-human TIM3 (clone 344823)	BD Biosciences	747961
Anti-human PD-1 (clone EH12.2H)	BioLegend	329952
Anti-human FoxP3 (clone PCH101)	eBioscience/	17-5773-82
	ThermoFisher	
Anti-human Granzyme B (clone	Invitrogen	MA523639
351927)		
Anti-human CD107a (clone H4A3)	BioLegend	328641
Anti-human Perforin (clone B-D48)	BioLegend	353312
Mouse anti-human LY6G6D (clone	BioLegend	367004
13.8)		
Mouse IgG1kappa (clone MOPC-21)	BioLegend	400114
Anti-LY6G6D	BioLegend	367003
Mouse IgG1 control antibody	Dako	X0931
Critical Commercial Assays		
Zombie Green Fixable Viability Dye	BioLegend	423112
Zombie NIR Fixable Viability Dye	BioLegend	423106
CellTrace [™] Violet Dye	Invitrogen	C34557
CellTrace [™] FarRed	Invitrogen	C34564
CellTrace [™] Oregon Green [™] 488	ThermoFisher Scientific	C34555
EasySep [™] Human T Cell enrichment	StemCell Technologies	19051
Kit negative selection		
Steady-Glo® Luciferase Assay System	Promega	E2520

Buffer Set CellTriter 96® AQueous One Solution Cell Proliferation Assay Cytotoxicity detection kit-Plus Cell Line Nucleofector™ Kit V Amaxa/Lonza VCA-1003 Phospholipase C Protein, Phosphatidylinositol-Specific QIFIKI™ Dako CORNING TRANSWELL-96 SYSTEM, 4umPC MEMBRANE AccuCheck COUNTING beads DMEM-F12, HEPES Gibco Minimum Essential Medium Eagle MisibaseF™ Misiba	Foxp3 / Transcription Factor Staining	eBioscience/	00-5523-00
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	plasmid		
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FlowJo V10 FlowJo, LLC	FlowJo V10	FlowJo, LLC	
GraphPadPrism 8.0/9.0 GraphPad Software	GraphPadPrism 8.0/9.0	GraphPad Software	
Ensight Software Perkin Elmer	Ensight Software	Perkin Elmer	
SoftMax Pro (ELISA reader)	SoftMax Pro (ELISA reader)		

Table 1. Cell lines:

Cell line	Source	Culture method
CL-14	Leibniz Institute DSMZ Cat#:	DMEM/F-12-20%
	ACC 504	
HT55	Public Health England	EMEM-20
	cat.nr: 85061105	
	Lot: 09K002	
NCI-H508	ATCC	RPMI-10
LS1034	ATCC-CRL-2158	RPMI-10
NCM460	Incell	M3-base medium-10%
	lot#C2010AUG10-01	
HEK 293	ATCC CRL-1573	EMEM-10
Rec. 293 LY6G6D+	Own description	DMEM-10 + 1 mg/ml G418
MDST8	ECACC	DMEM-10
	Lot: WT13K0001	
SK-CO1	ATCC HTB-39	EMEM-10
	lot: 61834531	
Jurkat NFAT luc	BI Canada, Laval	RPMI-10 (0.25 mg/ml
		Hygromycin)