Supplemental material

SUPPLEMENTAL METHODS

Cloning

Amplified ITGB6 and pLenti CMV GFP Blast vector were digested with BamHI (NEB; R3136) and SalI (NEB; R3138) restriction enzymes and ligated with Quick ligation kit (NEB; M2200). Control vector was constructed by ligating a short non-coding oligonucleotide instead of the amplified ITGB6 gene.

Primers containing BamHI and SalI restriction sites for PCR amplification of ITGB6:

TAAGGATCCGCCACCATGGGGATTGAGCTGGTCTG

TAAGTCGACAGCGGCCTACCCATCTGAGGAAAGGCC

Lentivirus production and viral transduction

In a T75 cell culture flask, HEK 293T cells were transfected with 9 µg ITGB6 expression vector, 4 µg pMD2.G (Addgene plasmid # 12259) and 7 µg pCMV-dR8.91 (Addgene vector database # 2221) using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturers protocol for lentiviral production. After 6h, transfection medium was removed and 10 ml fresh growth medium added. Supernatant containing the viral particles was harvested 24h and 48h later, filtered with 0.45 µm pore size, and frozen at -80°C.

CT26 and MC38-GFP cells (donated by Prof. Lubor Borsig, Institute of Physiology, University of Zurich, Zurich) were seeded at ~60% confluency and transduced by adding viral supernatant together with 8 µg/ml Polybrene (Merck; H9268). After 24h viral medium was removed and fresh growth medium added. 48h after initiation of transduction blasticidine selection was started by adding fresh medium containing 5 µg/ml blasticidine (Merck; 15205) and continued until untransduced control cells were completely dead.

Proliferation assay

In 96-well plates, MC38-ITGB6 and MC38-Ctrl as well as CT26-ITGB6 and CT26-Ctrl cells were seeded at 125 cells per well. After 24h (d0) or 96h (d3), cell number was defined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega G7571) according to manufacturer's instructions and measured using BioTek Synergy 2 Microplate Reader.

Western Blot

Cells were lysed in RIPA cell lysis buffer (25 mM Tris–HCl pH 7.6, 150 mM sodium chloride, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with cOmplete™ Mini Protease Inhibitor Cocktail (Merck; 11836153001). Protein extracts (20 µg) were run on 10% polyacrylamide gels, transferred to nitrocellulose membranes and visualized by immunoblotting with ITGB6 antibody (R&D Systems; MAB2389) and anti-mouse IgG-HRP (Santa Cruz; sc-2005).

Co-Immunoprecipitation

Cells were lysed in IP lysis buffer (20mM Tris-HCL pH 7.5, 150mM sodium chloride, 1% IGEPAL, 5mM magnesium chloride) with cOmplete™ Mini Protease Inhibitor Cocktail (Roche; 04693132001) and sonicated. Protein extracts (1 mg) were incubated overnight with protein A beads fast flow (Cytiva; 17528001) pre-coated with 2 µg ITGB6 antibody (R&D Systems; AF4155) at 4°C. Beads were washed with IP Wash buffer (20mM Tris-HCL pH 7.5, 150mM sodium chloride, 0,1% IGEPAL, 5mM magnesium chloride) and taken up in Laemmli buffer. Eluted samples were applied to 12,5% polyacrylamide gels transferred to nitrocellulose membranes and visualized by immunoblotting with ITGB6 antibody (R&D Systems; AF4155), ITGA antibody (abcam, ab179475), anti-rabbit IgG-HRP (Dako; P0448) and anti-sheep IgG-HRP (R&D Systems; HAF016).

Tumor cell injections

CT26-ITGB6 and CT26-Ctrl tumor cells were suspended in DMEM high glucose cell culture medium mixed 1:1 with matrigel (Corning 354263) and 60’000 cells were injected into the cecum wall (orthotopic model) or 100’000 cells were injected subcutaneously into the flanks (heterotopic model). MC38-ITGB6 and MC38-Ctrl tumor cells were suspended in DMEM high glucose cell culture medium mixed 1:1 with matrigel and 300’000 cells were injected subcutaneously into the flanks of the mice. 4T1 breast cancer cells (ATCC® CRL-2539™) were suspended in RPMI 1640 cell culture medium mixed 1:1 with matrigel and 100’000 cells subcutaneously injected subcutaneously into the flanks of the mice.

Histology

For histological studies, mouse tumors were fixed in 4% formalin, dehydrated by a graded series of ethanol (70 to 100%) and embedded in paraffin wax. 5 µm sections were cut using a rotary microtome

(Zeiss Hyram M 15). To further process the samples, the tissue sections were deparaffinized with Histo Clear (Chemie Brunschwig/National Diagnostics, HS-200) and rehydrated using a graded series of ethanol (100% to 70%).

For mouse IHC stainings, the rehydrated samples were heated (98 C°) for 30 min in antigen retrieval solution (Dako; S169984; Target Retrieval Solution; pH: 6.0). Endogenous peroxidases were blocked for 15 minutes with 0.9% H_2O_2 in PBS and unspecific antibody binding blocked by incubation with 2.5% horse serum for 1 hour. Primary antibodies CD8 (Cell Signaling; CD8α (D4W2Z) XP® Rabbit mAb #9894), CD4 (Cell Signaling; CD4 (D7D2Z) Rabbit mAb #25229), CD3 (Abcam; Anti-CD3 ab5690), Ki67 (Fisher Scientific; 12693697, RM-9106-S), pSmad2 (Cell Signaling; Phospho-Smad2 (Ser465/467) (138D4) #3108), pSmad3 (Abcam; Anti-Smad3 (phospho S423 + S425) [EP823Y] ab52903), CALD1 (Merck; Anti-CALD1 HPA008066), IGFBP7 (Merck; Anti-IGFBP7 HPA002196), SOX4 (Abcam; Anti-SOX4 ab86809) were incubated at 4 °C overnight. On the next day, the slides were incubated for 1 hour with the secondary antibody (Vector Laboratories; VC-MP-7401-L050 ImmPRESS™ anti-Rabbit IgG HRP; 1 drop) at room temperature followed by 1 minute DAB staining (Vector Laboratories; VC-SK-4105-L120; DAB ImmPACT™ DAB Peroxidase Substrate brown). Lastly, the sections were counterstained for 10 seconds with hematoxylin (Schleicher&Schuell; 10311651), dehydrated and mounted. The processed samples were examined under a light microscope (Zeiss Imager. Z2) and images were taken at 10X magnification. Quantification of positive cells was performed using Image J Software, 1.52a.

For IF stainings, the rehydrated samples were heated (98 C°) for 30 min in antigen retrieval solution (Dako; S236784; Target Retrieval Solution; pH: 9.0). Unspecific antibody binding was blocked by incubation with 2.5% horse serum for 1 hour. Primary antibodies CD8 (Thermo Fisher Scientific; CD8a (4SM15) 14-0808-82) and pSmad3 (Abcam; Anti-Smad3 (phospho S423 + S425) [EP823Y] ab52903), were incubated at 4 °C overnight. On the next day, the slides were incubated for 1 hour with the secondary antibodies (Thermo Fisher Scientific; Goat anti-rat Alexa Fluor 594, A11007) (Thermo Fisher Scientific; Goat anti-rabbit Alexa Fluor 647, A21244) at room temperature, washed and mounted. The processed samples were examined under a light microscope (Zeiss Imager. Z2) and images were taken at 20X or 40X magnification.

For human IHC stainings, archived formalin-fixed, paraffin-embedded human colorectal carcinoma tissue was cut in 4-µm sections using a rotary microtome (Zeiss HM 355S). The sections were

deparaffinised at 60°C overnight and rehydrated using a graded series of ethanol (100% to 70%). The rehydrated samples were heated in antigen retrieval solution (Dako; S2369; Target Retrieval Solution; pH: 6.0) in a water bath for 20 minutes at 95°C. Endogenous peroxidases were blocked by 7,5% H2O2 in demineralized water for 10 minutes and endogenous biotin was blocked with an Avidin/Biotin Blocking kit (Biozol; # SP-2001). Subsequently, the sections were incubated with primary Sox4 antibody (Invitrogen; PA5-41442) diluted 1:70 in 2,5% normal horse serum for 60 minutes at room temperature. The sections were incubated with Biotinylated Antibody for 30 minutes followed by the ABC reagent for 30 minutes at room temperature (Horse anti-Rabbit IgG Vectastain Elite ABC-Kit; Biozol #PK-7200). The immunodetection was performed with the NovaRed substrate kit (Vector; #SK-4800) for 4 minutes at room temperature. The sections were counterstained with Haematoxylin (Vector; H-3401), dehydrated and mounted in Vectamount Permanent Mounting Medium (Vector; # H-5000). The processed samples were examined under a light microscope (Leica CTR 6000) and images were taken at 20X magnification.

RT-qPCR analysis of mouse tumor

Tumor tissues (0.5 cm) were homogenized using gentleMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) and RNA isolated using the Maxwell RSC simplyRNA Tissue kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNA concentration was measured using absorbance at 260 and 280 nm. Complementary DNA (cDNA) was synthesized using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. RT-PCR was performed using FAST qPCR Master Mix and pre-designed Taqman assays (Thermo Fisher Scientific, USA) on a QuantStudio 6 system using the QuantStudio software (Thermo Fisher Scientific, USA). Mouse GAPDH or β-actin were used as endogenous controls. Relative expression levels were calculated according to the ΔΔCT method and samples were measured in triplicates.

RT-qPCR analysis of human tumor specimen

Total RNA was isolated using a fully automated extraction method from FFPE tissue sections (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) as described previously [1, 2]. *ITGB6* was detected using a TaqMan™ Gene Expression Assay (Thermo Fisher Scientific #4351368) according to the manufacturers instructions. Additional

controls for the detection of the housekeeping gene (RPL37A) and absence of DNA contamination (PAEP) were performed as previously described [1, 2]. The SuperScript III Platinum One-Step Quantitative RT-PCR-System with ROX (LifeTechnologies) was used as recommended by the manufacturer except for a prolonged reverse transcription time of 30 min at 50°C. A reaction (10 µl total volume) consisted of 0.2 µl RT/Taq-mix, 50 nM ROX Reference Dye (Life Technologies), 10 ng total RNA, 500 nM forward/reverse primer each and 250 nM probe. The reactions were assayed in using a Mx3005P qPCR system (Agilent) together with the Versant kPCR software (Siemens Healthcare Diagnostics). For quantification of RNA samples the absence of residual DNA was analyzed by DNA-specific primers for the *progestagen-associated endometrial protein (PAEP)* gene and was in all cases negative. On each plate a commercial qPCR human reference total RNA (qRef, Agilent) was used as a positive control and a non-template control (NTC) as negative control. The fluorescence threshold (ROX dRn) was set to 0.02 for all samples. All samples were normalized using RPL37A (mean of triplicates) as a reference gene. Subsequently, the ΔΔCTmethod was used for calculation of the respective fold changes for each target gene.

The sequences of primers and probes were as follows:

RPL37A forward: 5′-TGTGGTTCCTGCATGAAGACA-3′, reverse: 5′-GTGACAGCGGAAGTGGTATTGTAC-3′, probe: 5′-Fam-TGGCTGGCGGTGCCTGGA-BHQ1-3′; PAEP forward: 5′-CACAGAATGGACGCCATGAC-3′, reverse: 5′-AAACCAGAGAGGCCACCCTAA-3′, probe: 5′-Fam-AAGCCCTCAGCCCTGCTCTCCATC-BHQ1-3′.

SUPPLEMENTAL DATA

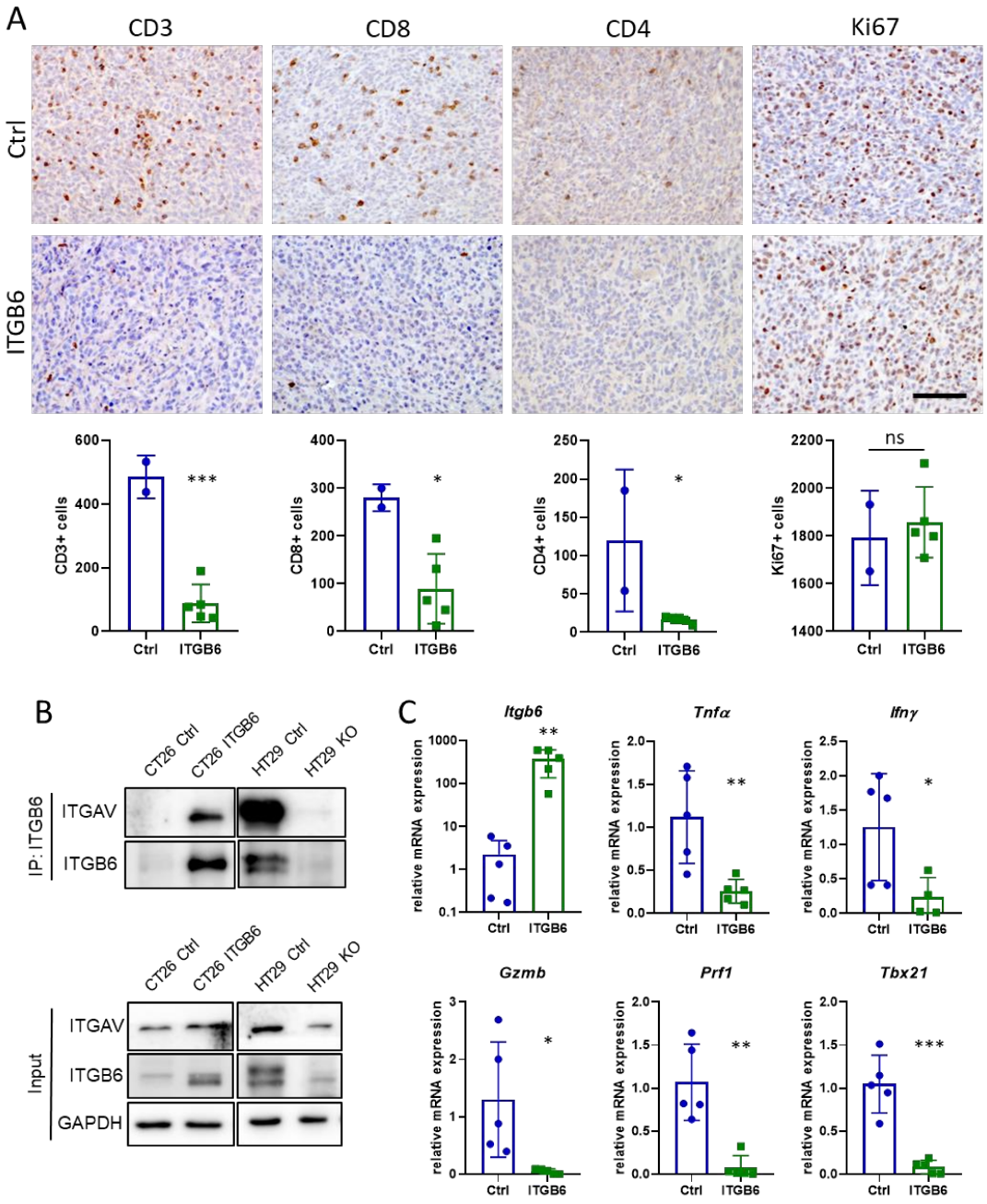

Fig. S1. (A) IHC stainings of CD3, CD4, CD8 and Ki67 in CT26-ITGB6 and CT26-Ctrl cecum tumors. Representative images of IHC stainings (top) and quantification of the number of stained cells (bottom) (n=2 (Ctrl), 5 (ITGB6)). (B) Co-immunoprecipitation of ITGAV and ITGB6. Immunoprecipitation with ITGB6 antibody (top) and expression of the input cells (bottom) HT29 knockout (KO) cells as technical control. (C) RT-qPCR analysis of anti-tumor immune mediators in tumor tissue of CT26-ITGB6 and CT26-Ctrl cecum tumors (n=5). Scale bar = 100 µm. Means and SDs are shown. Unpaired two-tailed t-tests were used to calculate statistical significance. ns = not significant ($p \geq 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

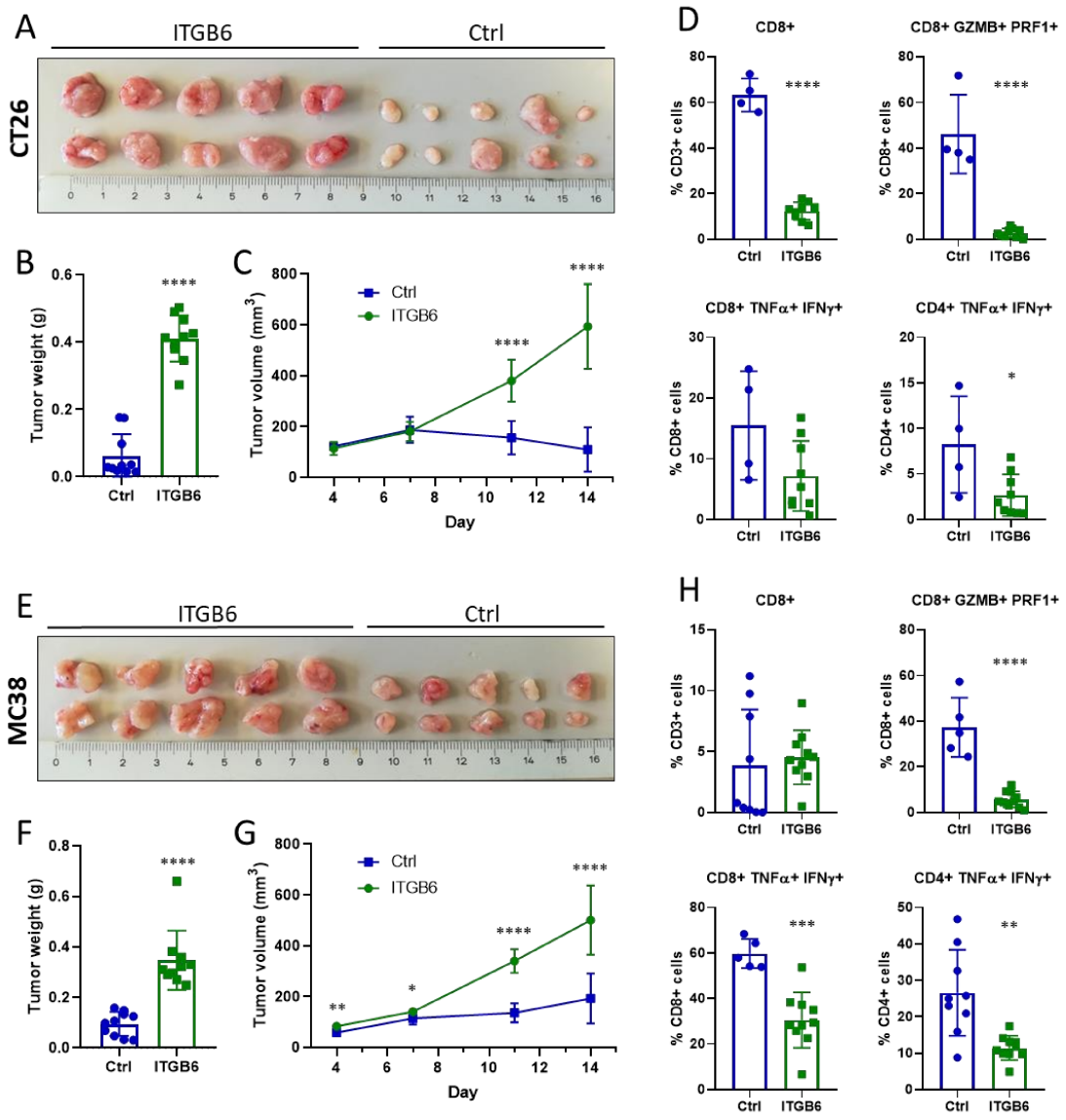

Fig. S2. (A) Subcutaneous CT26-ITGB6 and CT26-Ctrl tumors at day 14 after injection into Balb/c mice. (B) Weight of CT26-ITGB6 and CT26-Ctrl tumors at day 14 after injection. (C) Tumor volume development of CT26-ITGB6 and CT26-Ctrl tumors. (D) Flow cytometry analysis of T-cells isolated from CT26-ITGB6 and CT26-Ctrl tumors. (E) Subcutaneous MC38-ITGB6 and MC38-Ctrl tumors at day 14 after injection into C57BL/6 mice. (F) Weight of MC38-ITGB6 and MC38-Ctrl tumors at day 14 after injection. (G) Tumor volume development of MC38-ITGB6 and MC38-Ctrl tumors. (H) Flow cytometry analysis of T-cells isolated from MC38-ITGB6 and MC38-Ctrl tumors. Means and SDs are shown (n=5 mice, 2 tumors per mouse). Unpaired two-tailed t-test (B, D, F, H) and two-way ANOVA with Tukey's post-hoc test (C and G) were used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

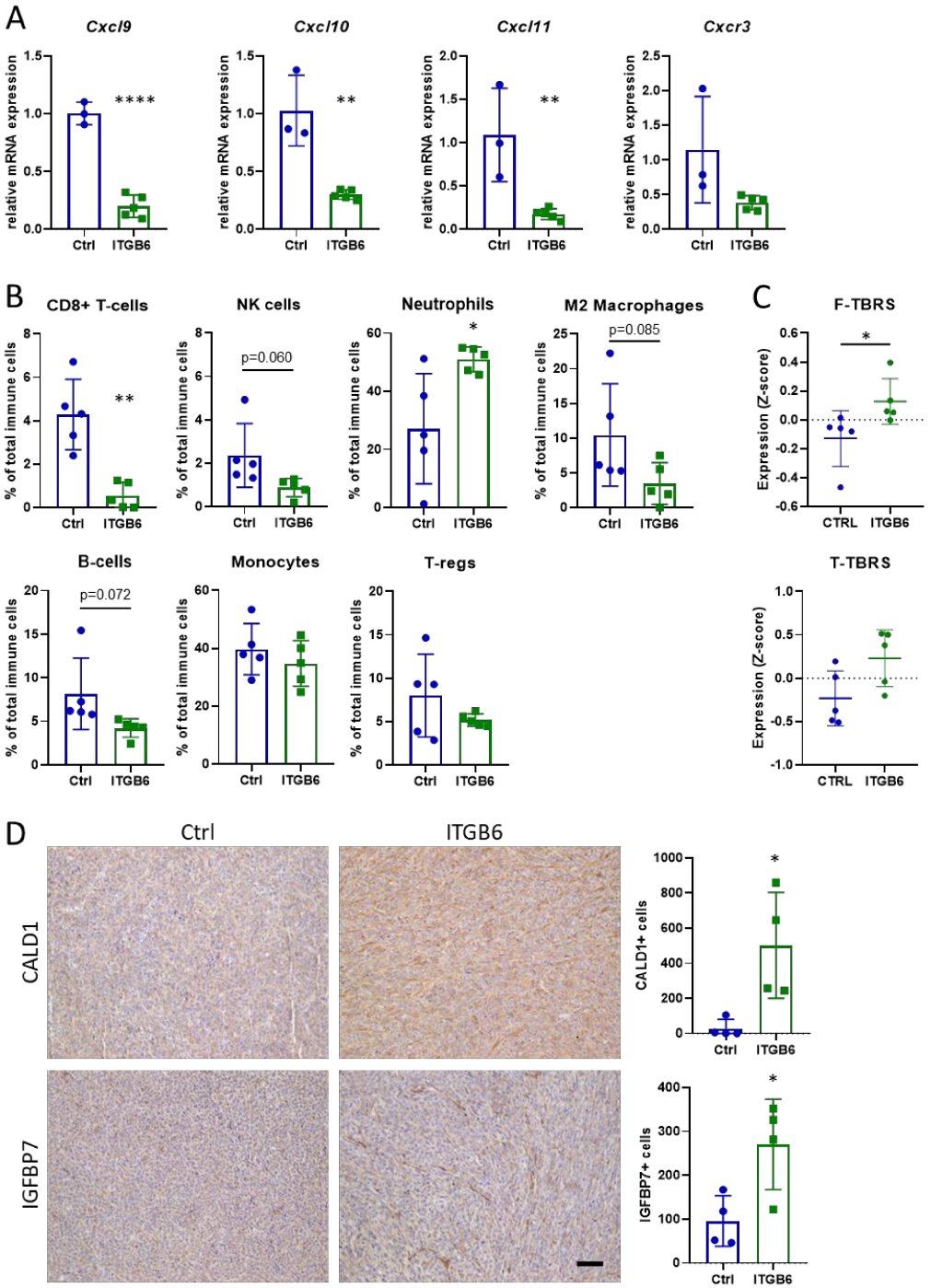

Fig. S3. (A) RT-qPCR analysis of subcutaneous CT26-ITGB6 and CT26-Ctrl tumors. (B) Deconvolution of immune cell type proportions from the bulk tissue RNA-seq data of MC38-ITGB6 and MC38-Ctrl tumors based on a reference expression signature panel. (C) Expression levels of TBRS in fibroblasts (F-TBRS) and T-cells (T-TBRS) in MC38-ITGB6 and MC38-Ctrl tumors (D) IHC stainings of CALD1 and IGFBP7 in subcutaneous CT26-ITGB6 and CT26-Ctrl tumors. Representative images of IHC stainings (left) and quantification of the number of stained cells (right). Scale bar = 100 µm. Means and SDs are shown (n=5). Unpaired two-tailed t-tests were used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

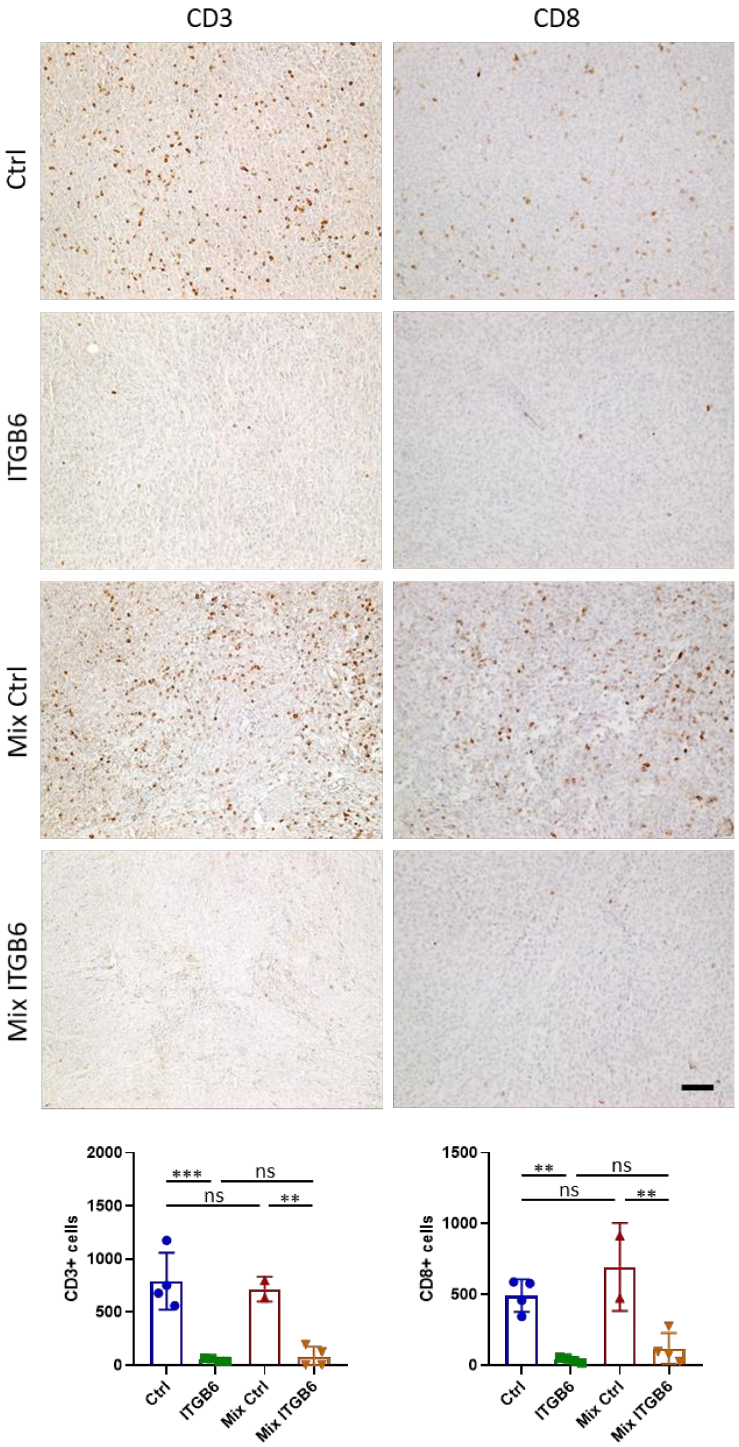

Fig. S4. IHC stainings of CD3 and CD8 in subcutaneous CT26-Ctrl tumors or CT26-ITGB6 tumors injected in both flanks or CT26-Ctrl tumors in one flank and CT26-ITGB6 tumors in the other flank of the mice (Mix). Representative images of IHC stainings (top) and quantification of the number of stained cells (bottom). Scale bar = 100 µm. Means and SDs are shown (n=2-5). One-way ANOVA with Tukey's post-hoc test was used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

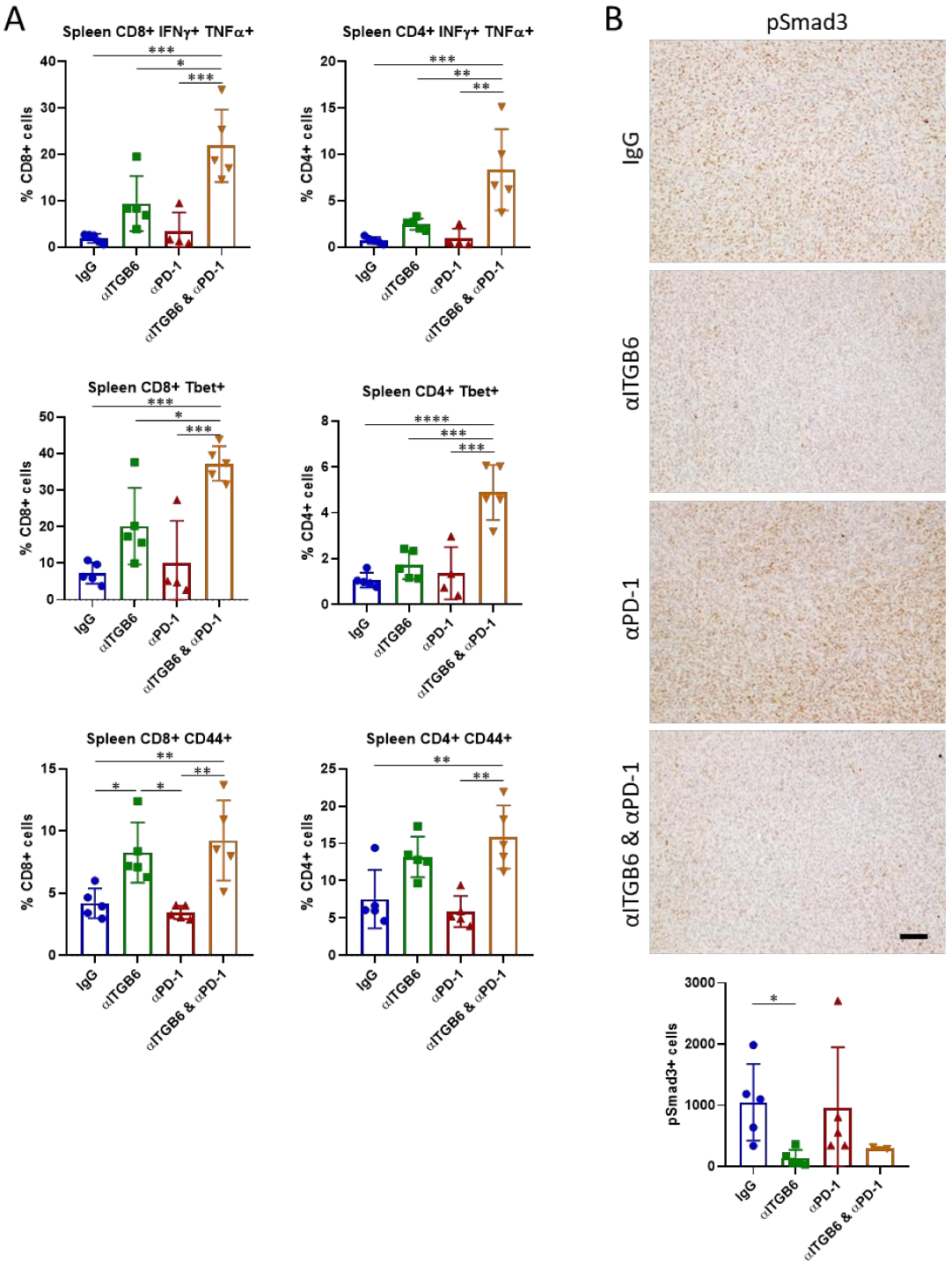

Fig. S5. (A) Flow cytometry analysis of splenic CD8+ and CD4+ T-cells in mice bearing CT26-ITGB6 tumors treated with αITGB6 (6.8G6), αPD-1, αITGB6 (6.8G6) & αPD-1 or IgG control antibody. (B) IHC staining of pSmad3 in subcutaneous CT26-ITGB6 tumors treated with αITGB6 (6.8G6), αPD-1, αITGB6 (6.8G6) & αPD-1 or IgG control antibody. Representative images of IHC staining (top) and quantification of the number of stained cells (bottom). Scale bar = 100 µm. Means and SDs are shown (n=5 mice). One-way ANOVA with Tukey's post-hoc test (A) and unpaired two-tailed t-test (B) was used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

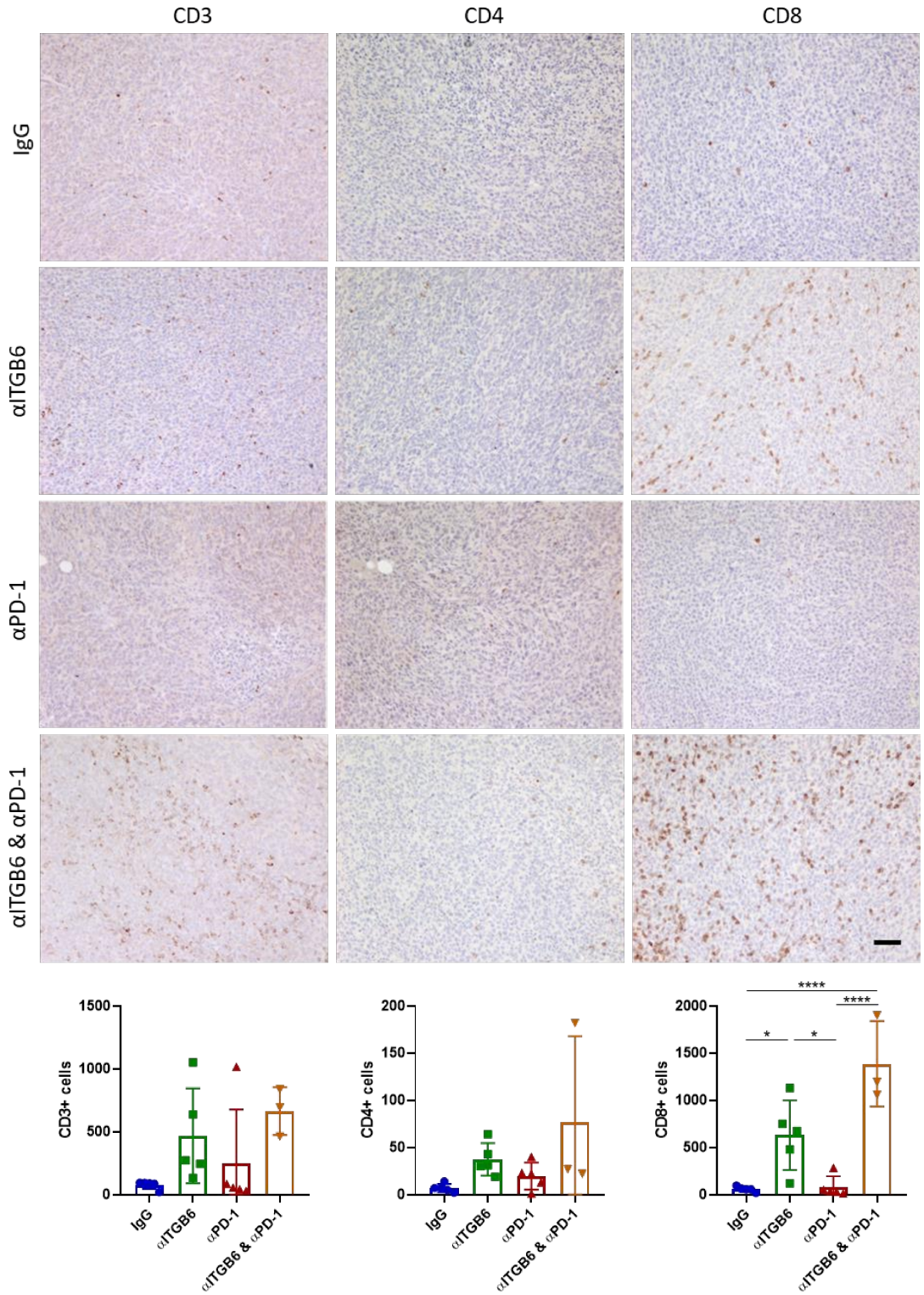

Fig. S6. IHC stainings of CD3, CD4 and CD8 in subcutaneous CT26-ITGB6 tumors treated with αITGB6 (6.8G6), αPD-1, αITGB6 (6.8G6) & αPD-1 or IgG control antibody. Representative images of IHC stainings (top) and quantification of the number of stained cells (bottom). Scale bar = 100 µm. Means and SDs are shown (n=3-5). One-way ANOVA with Tukey's post-hoc test was used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

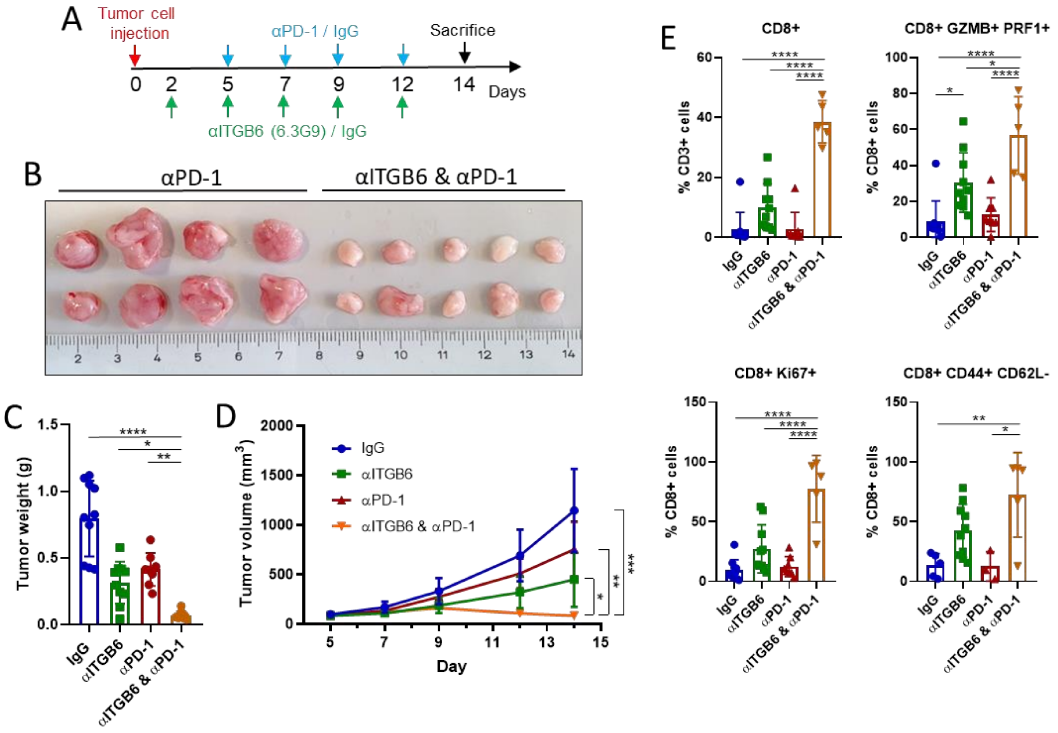

Fig. S7. (A) Experimental design of αITGB6 (6.3G9) & αPD-1 antibody administration. (B) Subcutaneous CT26-ITGB6 tumors treated with αPD-1 or αITGB6 (6.3G9) & αPD-1. (C) Tumor weight of subcutaneous CT26-ITGB6 tumors treated with αITGB6 (6.3G9), αPD-1, αITGB6 (6.3G9) & αPD-1 or IgG control. (D) Tumor volume development of subcutaneous CT26-ITGB6 tumors treated with αITGB6 (6.3G9), αPD-1, αITGB6 (6.3G9) & αPD-1 or IgG control. (E) Flow cytometry analysis of CD8+ T-cells in CT26-ITGB6 tumors treated with αITGB6 (6.3G9), αPD-1, αITGB6 (6.3G9) & αPD-1 or IgG control antibody. Means and SDs are shown (n=4-5 mice, 2 tumors per mouse). One-way ANOVA (C and E) and two-way ANOVA (D) with Tukey's post-hoc test was used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

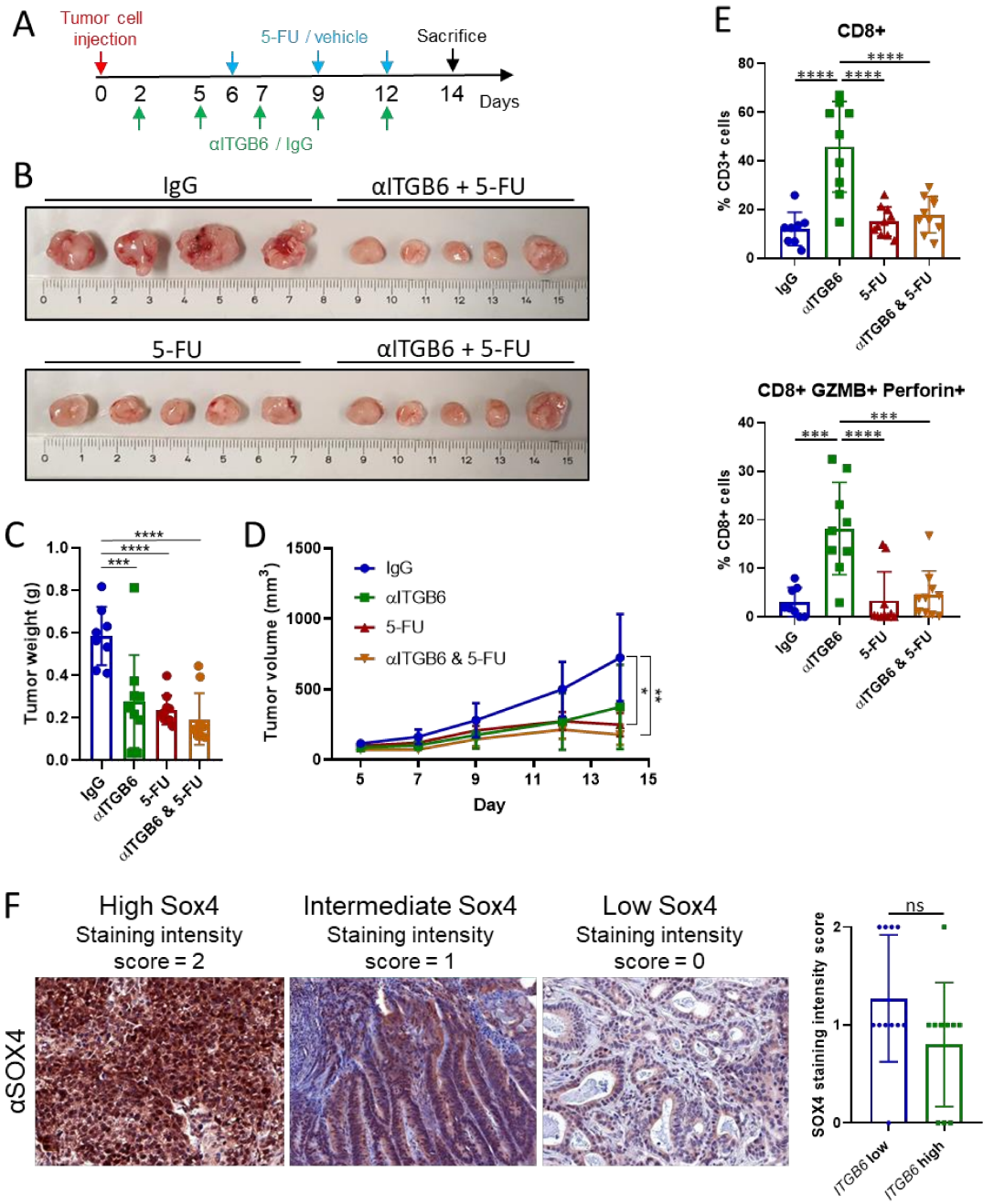

Fig. S8. (A) Experimental design of αITGB6 (6.8G6) & 5-FU treatment administration. (B) Representative image of subcutaneous CT26-ITGB6 tumors treated with 5-FU, αITGB6 & 5-FU or IgG control. (C) Tumor weight of subcutaneous CT26-ITGB6 tumors treated with αITGB6, 5-FU, αITGB6 & 5-FU or IgG control. (D) Tumor volume development of subcutaneous CT26-ITGB6 tumors treated with αITGB6, 5-FU, αITGB6 & 5-FU or IgG control. (E) Flow cytometry analysis of CD8+ T-cells in CT26-ITGB6 tumors treated with αITGB6, 5-FU, αITGB6 & 5-FU or IgG control antibody. (F) IHC staining of human CRC tumors with high (ITGB6: 40-ΔCt > 31.9) or low (ITGB6: 40-ΔCt < 28.4) ITGB6 mRNA expression. Representative images of staining intensity (left) and quantification of staining intensity scores (right). Means and SDs are shown (n=4-5 mice, 2 tumors per mouse). One-way ANOVA (C and E), two-way ANOVA (D) and Mann-Whitney test (F) was used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

	Patients, n = 343
Gender	
Males	201 (58.6 %)
Females	142 (41.4 %)
Age	
Median	70 years
Range	24 – 96 years
ITGB6	
40-ΔCt ≤ 30	109
40-ΔCt > 30	234
UICC stages	
Stage I	75 (21.87 %)
Stage II	117 (34.11 %)
Stage III	88 (25.66 %)
Stage IV	63 (18.37 %)
Histopathological grading	
Low grade (G_1/G_2)	226 (65.89)
High grade (G_3/G_4)	117 (34.11)

Table S1: Clinical characteristics of the colon carcinoma patients included in the analysis of ITGB6 expression

Supplier	Clone	Antigen	Fluorochrome
BioLegend	17A2	CD3	BV785
BD Biosciences	GK1.5	CD4	BV711
BioLegend	MP6-XT22	TNFa	BV650
BioLegend	30-F11	CD45	BV510
BioLegend	QA16A02	Granzyme B	PerCP-Cy5.5
BioLegend	XMG1.2	IFNg	PE-Cy7
Thermo Fisher Scientific	RA3-6B2	B220/CD45R	PE-Cy5
BD Biosciences	53-6.7	CD8	PE-CF594
BioLegend	S16009B	Perforin	APC
Thermo Fisher Scientific	4B10	Tbet	PE
BioLegend	RM4-5	CD4	BV650
BioLegend	29F.1A12	PD-1	APC
BioLegend	MEL-14	CD62L	FITC
BioLegend	UC10-4B9	CTLA4	PE-Cy7
Thermo Fisher Scientific	IM7	CD44	APC
BioLegend	16A8	Ki67	AF700

Table S2: Flow cytometry antibodies

References

1. Naschberger E, Liebl A, Schellerer VS, et al. Matricellular protein SPARCL1 regulates tumor microenvironment-dependent endothelial cell heterogeneity in colorectal carcinoma. J Clin Invest 2016;126:4187-4204.
2. Klingler A, Regensburger D, Tenkerian C, et al. Species-, organ- and cell-type-dependent expression of SPARCL1 in human and mouse tissues. PLoS One 2020;15:e0233422.