IL-10 dampens antitumor immunity and promotes liver metastasis via PD-L1 induction

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Fig. S1: II10-deficiency in mouse promotes primary tumor growth

(A) Schematic overview of the intracaecal injection of MC38 cancer cells for spontaneous liver metastasis induction. (B) Overall survival and (C) Primary tumor weight in the caecum of *Wt* and *II10-/-* mice ($n \ge 8$ mice per group). (D) Overall survival of the mice after intrasplenic injection of MC38 cells. Data are presented as mean \pm SEM. Non-significant (ns): p > 0.05; *: $p \le 0.05$; **: p < 0.01, as calculated by Mantel-Cox test or Mann-Whitney *U* test. p.i.: post injection



Fig. S2: The pathogenic role of IL-10 in liver metastasis formation is independent of colitis severity

(A) Schematic overview of the intrasplenic injection of MC38 cancer cells for forced liver metastasis induction in *II10+/+* and *II10-/-* littermates (n = 6 mice per group). (B) Representative pictures of liver metastasis, as well as analysis of liver weight and number of liver metastases. (C) Representative endoscopic view on day 21 and colitis score at different time points during liver metastasis formation. (D) Schematic overview of the i.s. injection of MC38 cancer cells for forced liver metastasis induction using *II10+/+* and *II10-/-* littermates following fecal microbiome (MB) transplant (*MB1, MB2*) (n ≥ 10 mice per group). (E) Representative endoscopic view of colon on day 21 post injection (i.s.) and (F) Colitis severity score at different time points during the whole

procedure. Livers were harvested and (**G**) liver weight as well as (**H**) number of microscopic liver metastases were analyzed. Scale bar: 2 mm. Data are presented as mean \pm SEM. Non-significant (ns): p > 0.05; *: p ≤0.05; **: p < 0.01, as calculated by Mann-Whitney *U* test or by one-way ANOVA with Bonferroni post hoc tests. FMT: fecal microbiome transfer.



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Fig. S3: Treg expansion together with a dynamic IL-10 increase are observed during liver metastasis formation

(A) IL-10 expression in hematopoietic and nonhematopoietic cells of a healthy mouse liver. (B) $Foxp3^{RFP}$; $II10^{GFP}$ reporter mice received MC38 colon cancer cells i.s. and were sacrificed at the indicated time points (n ≥ 3 mice per group). (C) Frequency of Tregs within CD4+ T cells along metastasis formation. (D) General distribution of CD45+IL-10+ cells at the indicated timepoints. (E) IL-10 producing Tregs along metastasis formation. (F) *Wild type* and *DEREG* mice received i.s. of MC38 cells and livers were harvested in 21 days post injection. Liver weight (left) and number of liver metastasis (right) in mice with Treg depletion and their controls. Data are presented as mean ± SEM. Non-significant (ns): p > 0.05; *: p ≤ 0.05; **: p < 0.01; ***: p <0.001, as calculated by one-way ANOVA with Bonferroni post hoc tests.



Fig. S4: Innate cells are the major source of IL-10 producing cells in CRC

(A) Schematic overview of the intracaecal injection of MC38 cancer cells for CRC induction in *Foxp3*^{*RFP*};*II10*^{*GFP*} reporter mice (n ≥ 5 mice per group). Immune cells from cecum were then isolated and stained for flow cytometry 35 days post injection. The frequency of IL-10+ cells in the fraction of (B) CD45+ cells, (C) CD3- and T cells, and in (D) CD8+ T cells and CD4+ T cells was analyzed. (E) IL-10 expression in (F) Foxp3-IL-10+ T cells and (G) Foxp3+Tregs. (H) General distribution of all IL-10 producing CD45+ cells in healthy cecum and cecum tumor. Data are presented as mean ± SEM. Non-significant (ns): p > 0.05; *: p ≤ 0.05; **: p < 0.01; ***: p < 0.001, as calculated by Mann-Whitney *U* test.



Fig. S5: Different immune cell compositions in CRC and CRLM. Immune cells were isolated from human CRC and CRLM, and subsequently stained for flow cytometry. **(A)** Gating strategy and analysis for B cells, innate cells and T cells in **(B)** CRC and **(C)** CRLM. **(D, E)** Representative FACS plots and analysis of CD4+ and CD8+ T cells in (D) CRC and (E) CRLM. **(F, G)** Representative FACS plots and analysis of CD4+CD25+ T cells in (D) CRC and (E) CRLM. Data are presented as mean ± SEM. Non-significant (ns): p > 0.05; *: $p \le 0.05$; **: p < 0.01; ***: p < 0.001, as calculated by Mann-Whitney *U* test.





Fig. S6: IL-10 signaling in cancer cells and LSECs does not affect liver metastasis formation

(A) mRNA expression of the IL-10 and IL-10 receptor complex in MC38 and LLC cancer cells. (B) MFI of the IL-10Ra expression in MC38 and LLC cancer cells measured using flow cytometry. (C) mRNA expression of IL-10 and IL-10 receptor complex in total liver and LSECs isolated from Wt mice. (**D**) Quantification (Δ MFI) of IL-10Ra expression in total immune cells, LSECs and the rest of the cells isolated from Wt murine liver. (E) Phosphorylation of STAT3 in MC38 and LLC cancer cells upon in *vitro* IL-6 (10 ng/µl) or IL-10 (10 ng/µl) stimulation at indicated time points measured using Western Blot. (F, G) Phosphorylation of STAT3 in Wt LSECs after in vitro IL-10 stimulation (10 ng/µl) measured using (F) flow cytometry (after a 60-minute stimulation) or (G) Western blot (at indicated timepoints). (H) MC38-GFP cancer cells were intrasplenically injected into II10+/+ and II10-/- littermates (n \geq 7 mice per group). (I) Representative FACS-plot of extravasated MC38-GFP cells and (J) the number of extravasated MC38-GFP cells. (K) MC38 colon cancer cells were i.s. injected into mice with LSECs-specific IL-10Ra deletion and their littermate controls ($n \ge 12$ mice per group). (L) Representative images, liver weight as well as number of liver metastasis were analyzed. (M) Representative FACS plot and (N) ΔMFI quantification of pSTAT3 staining in hepatic immune cells isolated from Wt mice upon IL-10 (10 ng/µl) or IL-6 (10 ng/µl) stimulation in vitro. (O) Hepatic Treg frequency within CD4+ T cells and (P) //10 level measured using gPCR upon IL-10 and/or antiCD3/CD28 stimulation in vitro. Scale bar: 2 mm. Data are presented as mean ± SEM. Nonsignificant (ns): p > 0.05; ***: p < 0.001, as calculated by Mann-Whitney U test or oneway ANOVA (Bonferroni) with Bonferroni post hoc tests. LSECs: liver sinusoidal endothelial cells.



Fig. S7: PD-L1 deficiency enhances antitumor immunity of CD8+ T cells and reduces liver metastasis

(A) Bulk sequencing analysis on CD11b+ cells. (B) Gating strategy for Figure 6B. (C) Gating strategy for Figure 6C. (D to G) Pdl1+/+ and Pdl1-/- mice were i.s. injected MC38 cancer cells (n \geq 12 mice per group). (D) Livers were harvested 21 days post injection and metastatic burden was assessed. (E) 14 days post injection, representative FACS-plot and the frequency of hepatic CD8+ T cells, as well as

granzyme B level in CD8+ cells (**F**) before and (**G**) after coculture with MC38 cells *in vitro*. $n \ge 5$ mice per group. Scale bar: 2 mm. Data are presented as mean ± SEM. Non-significant (ns): p > 0.05; *: $p \le 0.05$; **: p < 0.01; ***: p < 0.001, as calculated by Mann-Whitney *U* test.

Supplementary materials and methods LSEC isolation

The mice were euthanized and liver perfusion was performed, first by flushing with PBS, and then with 5 mL 0.05% collagenase into the portal vein and vena cava. The liver was sliced into small pieces and digested with 1 mg/ml collagenase and 10 U/ml DNase at 37°C for 25 minutes in RPMI (10% FBS, 1% pen/strep), while shaking. The remaining liver was filtered through a 200-µm cell strainer and cell isolation was performed by centrifuging twice at 40 g for 4 minutes and once at 400 g for 20 minutes. An Optiprep (Sigma, Kawasaki, Japan) gradient was used to enrich LSECs from theliver. For isolating LSECs, MACS sorting with anti-CD146 magnetic antibodies was used according to the manufacturer's protocol. LSECs were cultured on collagen-coated plates for 5 days before stimulation.

Extravasation assay

To perform the extravasation assay, the forced liver metastasis model was used as described above. Here, the difference was the time of euthanization and modified cancer cells. MC38 cells with a green fluorescent protein (MC38-GFP) were used for injections, and livers were harvested after 24 h. The livers were cut, digested and smashed into single cell resolution as mentioned above. Then, the supernatant was collected twice after a 40 g centrifuge for 4 mins. Next, cells in 1:10 dilution were mixed with counting beads (Spherotech Inc) for flow cytometry. A mouse injected with MC38shC cells was used as a negative control.

Fecal microbiome transfer

Stool was collected either from *C57BL/6J* mice (MB1) or from *Rag-/-(Yale)* mice with a colitogenic microbiome (MB2) [1]. Dissolved stool in brain heart infusion medium (Millipore) was transplanted into the recipient mouse using a gavage needle.

Mouse colonoscopy

A colonoscopy was performed to determine the severity of the colitis. The degree of colitis (scale 0-15) was determined as published [2], where 0 represents no colitis and 15 represents severe colitis. The mice were under isoflurane anesthesia, and the colonoscopy (Karl Storz) was performed once a week. Colitis severity was scored by two blinded investigators.

Western Blot

Cells were lysed in lysis buffer on a plate, then scraped and centrifuged at 14,000 g for 5 min. The supernatant was transferred to a new tube. Protein concentration was assessed using the NanoDrop-instrument. Protein samples were equalized with water and Laemmli buffer (60 μ g/sample) and heated to 95°C for 5 min. Protein samples were run on a 10% Tris/Glyicine/SDS-PAGE, subsequently transferred from the gel to a PVDF-membrane using wet-blot electrophoresis for 60 min at 400 mA. The blotted membrane was incubated with blocking buffer (5% milk in PBS-T) for 1 hour, followed

by an incubation with antibody solution (1:1000 dilution) overnight at 4°C. The membrane was washed and incubated with the HRP-conjugated secondary antibody (1:2000) for 1 h. After washing, the blot was developed with chemiluminescent HRP substrate for 5 min before placing a film on the membrane (dark room). The photo film was run through a developer and the ladder was carefully marked on the film.

Bulk sequencing

To generate sequencing libraries, 2 mg RNA from each sample was used according to the manufacturer of NEBNext UltraTM RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). cDNA libraries were subsequently sequenced on Illumina HiSEquation 2500 yielding ~15 million 50 bp single-end reads per sample. To assess the RNA quality, FastQC v. 0.11.5 was used [3]. Alignment to mouse genome draft GRCm38.84 was conducted using STAR v. 2.5.0 [4]. For visualization and hierarchical clustering, using the transcripts per million method was used to normalize the reads, but raw read counts were used for differential expression analysis using DESeq2 v. 1.14 [5].

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Granzyme B Thermo Fisher Scientific Mm00442837 m1	10rb	Thermo Fisher Scientific	Mm00434157 m1	
	Granzvme R	Thermo Fisher Scientific	Mm00442837 m1	

Table S1: Taqman probes and primer sequences utilized for this study

Table S2: Flow cytometry antibodies utilized for this study

Antibody	Fluorochrom	company	Cat#	Clone	RRID
CD45	BV785	Biolegend	103149	30-F11	AB_2564590
CD45	BUV395	BD	564279	30-F11	AB_2651134
CD3	BV421	Biolegend	100228	17A2	AB_2562553
CD3	PE-Dazzle	Biolegend	100246	17A2	AB_2565883
CD3	BV650	Biolegend	100229	17A2	AB_11204249
CD3	BUV395	BD	740268	17A2	AB_2687927
CD4	APC	Biolegend	100412	GK1.5	AB_312697
CD4	BUV737	BD	612761	GK1.5	AB_2870092
CD8	PE-Cy7	Biolegend	100722	53-6.7	AB_312761
IL-10Ra	PE	Biolegend	112706	1B1.3a	AB_313519
Isotype	PE	Biolegend	400408	RTK2071	AB_326514
pSTAT3	BV421	Biolegend	651010	13A3-1	AB_2572088
PD-L1	BV711	Biolegend	124319	10F.9G2	AB_2563619
PD-1	BV421	Biolegend	135218	29F.1A12	AB_2561447
CD11b	PacBlue	Biolegend	101224	M1/70	AB_755986
CD11c	APC	Biolegend	117312	N418	AB_389328
Ly6G	AF488	Biolegend	127625	1A8	AB_2561339
Ly6C	PE	Biolegend	560592	AL-21	None availiable
CD19	APC-Cy7	Biolegend	115530	6D5	AB_830707
Fixable Viability Dye eFluor 506	BV510	Invitrogen	65-0866-14	ig doesnt target a spe	None availiable
PI	PE	Invitrogen	P1304MP	1	None availiable
Annexin	APC	Biolegend	640920	1	None availiable
TNFa	BV421	Biolegend	506328	MP6-XT22	AB_2562902
Granzym B	FITC	BioLegend	515403	GB11	AB_2114575