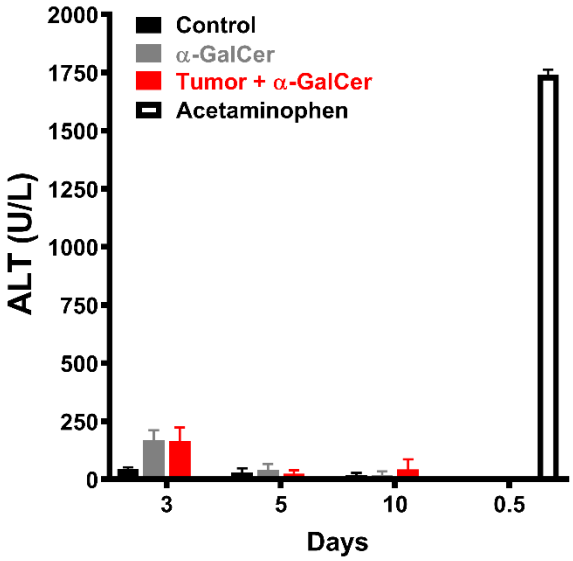


Figure S1. Liver injury assessment and representative livers from the survival study. Blood plasma ALT levels measured at day 3, 5 and 10 after repeated α-GalCer administration. Day3: Control (splenectomised mice) n=3 mice, α-GalCer n=3 mice, Tumor (CT26-iRFP) n=3 mice; Day5: Control: Control n=5 mice, α-GalCer n=6 mice, CT26-iRFP n=6 mice; Day10: Control n=3 mice, α-GalCer n=3 mice, CT26-iRFP n=3 mice; Data are represented as mean±s.e.m. Two-way Anova: no significant differences between treatment groups. Bar on the right shows ALT blood serum level 12h after Acetaminophen treatment (oral gavage) in C57BL/6J, n=3 mice.

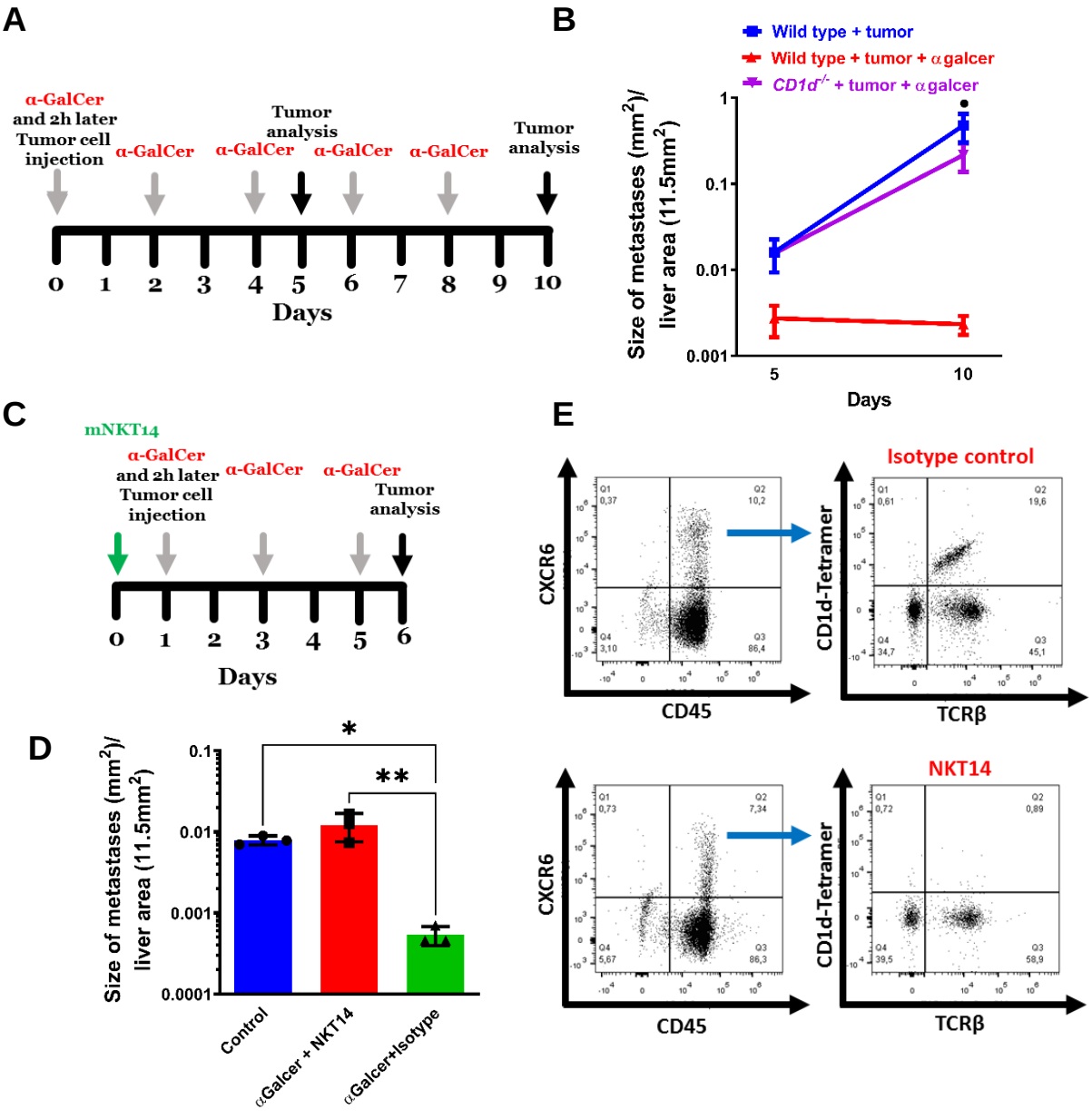
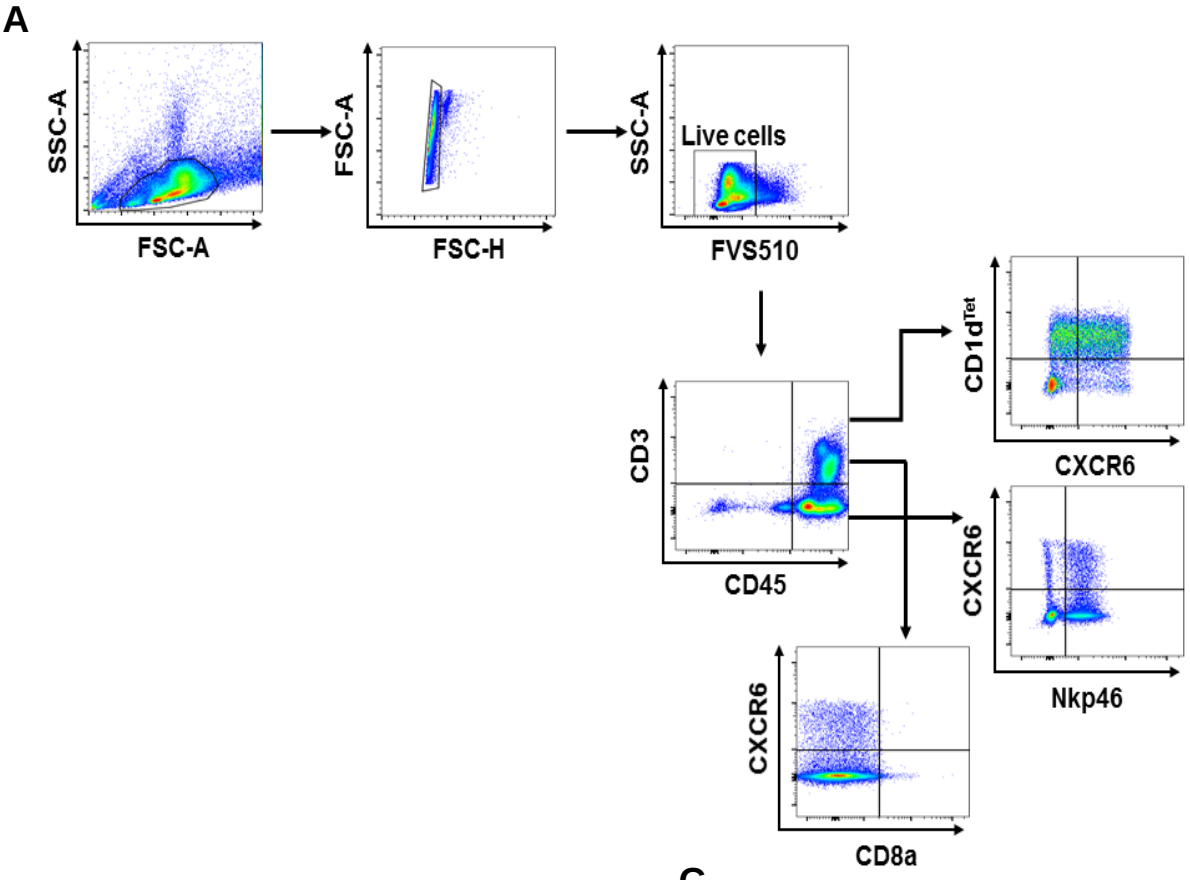
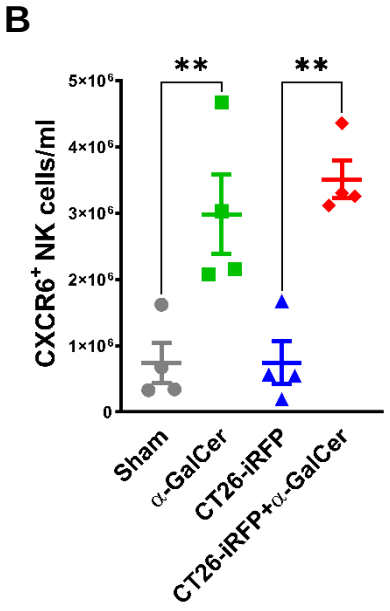

A
α-GalCer
and 2h later
Tumor cell
injection
α-GalCer
α-GalCer
Tumor
analysis
α-GalCer
α-GalCer
Tumor
analysis
0 1 2 3 4 5 6 7 8 9 10
Days
B
Wild type + tumor
Wild type + tumor + αgalcer
CD1d-/- + tumor + αgalcer
Size of metastases (mm2)/
liver area (11.5mm2)
1
0.1
0.01
0.001
5
10
Days
C
mNKT14
α-GalCer
and 2h later
Tumor cell
injection
α-GalCer
α-GalCer
Tumor
analysis
0 1 2 3 4 5 6
Days
D
Size of metastases (mm2)/
liver area (11.5mm2)
0.1
0.01
0.001
0.0001
*
**
Control
αGalcer + NKT14
αGalcer+Isotype
E
Isotype control
CXCR6
CD45
CD1d-Tetramer
TCRβ
Q1 0,37
Q2 10,2
Q4 3,10
Q3 86,4
Q1 0,61
Q2 19,6
Q4 34,7
Q3 45,1
NKT14
Q1 0,73
Q2 7,34
Q4 5,67
Q3 86,3
Q1 0,72
Q2 0,89
Q4 39,5
Q3 58,9

Figure S2. CD1d and iNKT cell dependent attenuation of liver metastases. **A,** Experimental timeline. **B,** Quantification of the size of liver metastases at day 5 and day 10 in CD1d deficient mice (purple line: day5 and day10 n=3 mice) and wild type mice after treatment with α-GalCer (red line: day5 n=6 mice, day10 n=8 mice) and in BALB/c wild type +tumor (CT26-iRFP) (blue line: day5 and day10 n=6 mice) mice, error bars show mean±s.e.m.; Two-way Anova with Šidák's multiple comparison: ⋆p<0.0066 at day10 BALB/c wild type tumor vs BALB/c wild type + tumor + α-GalCer). **C,** Timeline for α-GalCer treatment (light gray), iNKT cell depletion (mNKT14, green arrow) and metastatic tumor analysis (black arrows). **D,** Quantification of the size of liver metastases at day5 after tumor cell injection in untreated (blue bar n=3 mice) BALB/c wild type mice, mNKT14 and α-GalCer treated mice (red bar n=3 mice) and isotype control and α-GalCer treated mice (green bar n=3), error bars indicate s.e.m.; One-way Anova: ✶p<0.05 and ✶✶p<0.01. **E,** Representative dot blots showing iNKT cells in the liver after treatment of mice with isotype control and the depletion of iNKT cells ($TCR\beta^+$ CD1d-$tetramer^+$) after the treatment of the mice with NKT14.


A
SSC-A
FSC-A
FSC-A
FSC-H
SSC-A
Live cells
FVS510
CD3
CD45
CD1d^Tet
CXCR6
CXCR6
Nkp46
CXCR6
CD8a


B
**
**
CXCR6+ NK cells/ml
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
Sham
α-GalCer
CT26-iRFP
CT26-iRFP+α-GalCer

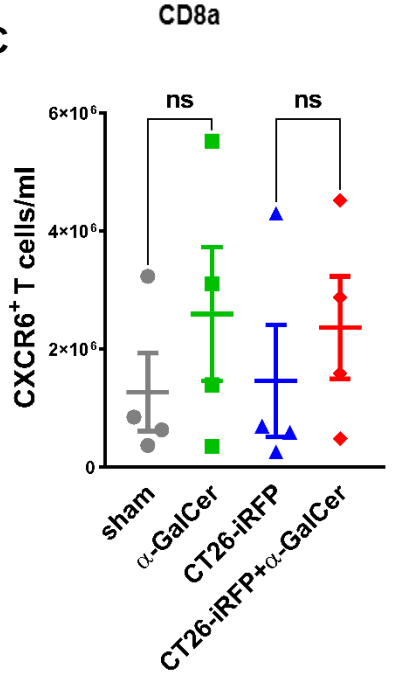

C
ns
ns
CXCR6+ T cells/ml
6×10^6
4×10^6
2×10^6
0
sham
α-GalCer
CT26-iRFP
CT26-iRFP+α-GalCer

Figure S3. Flow cytometric assessment hepatic of iNKT cells, NK cells and CD8 T cells after repeated α-GalCer treatment. **A,** Representative flow cytometry gating strategy to evaluate CXCR6-positive iNKT cells, NK cells and CD8 T cells in *Cxcr6*$^{gfp/+}$ mice at day3 after treatment. Leukocytes were identified based on size and granularity in liver cell suspensions. After exclusion of doublets and gating on live cells, iNKT cell were identified based on cell surface expression of CD45, CD3, CXCR6 and CD1d-tetramer positive staining. NK cells were CD45+, CD3-, CXCR6+ and Nkp46+. CD8 T cells were based on CD45, CD3, CXCR6 and CD8 cell surface expression. **B,** and **C,** show the quantification of NK cells (**B**) and CD8 T cells (**C**) in the liver at day 3 after repeated α-GalCer treatment. For all treatment groups n=4 mice, error bar show mean±s.e.m.; One-way Anova with Tukey's multiple comparisons test, ✷✷p<0.01.

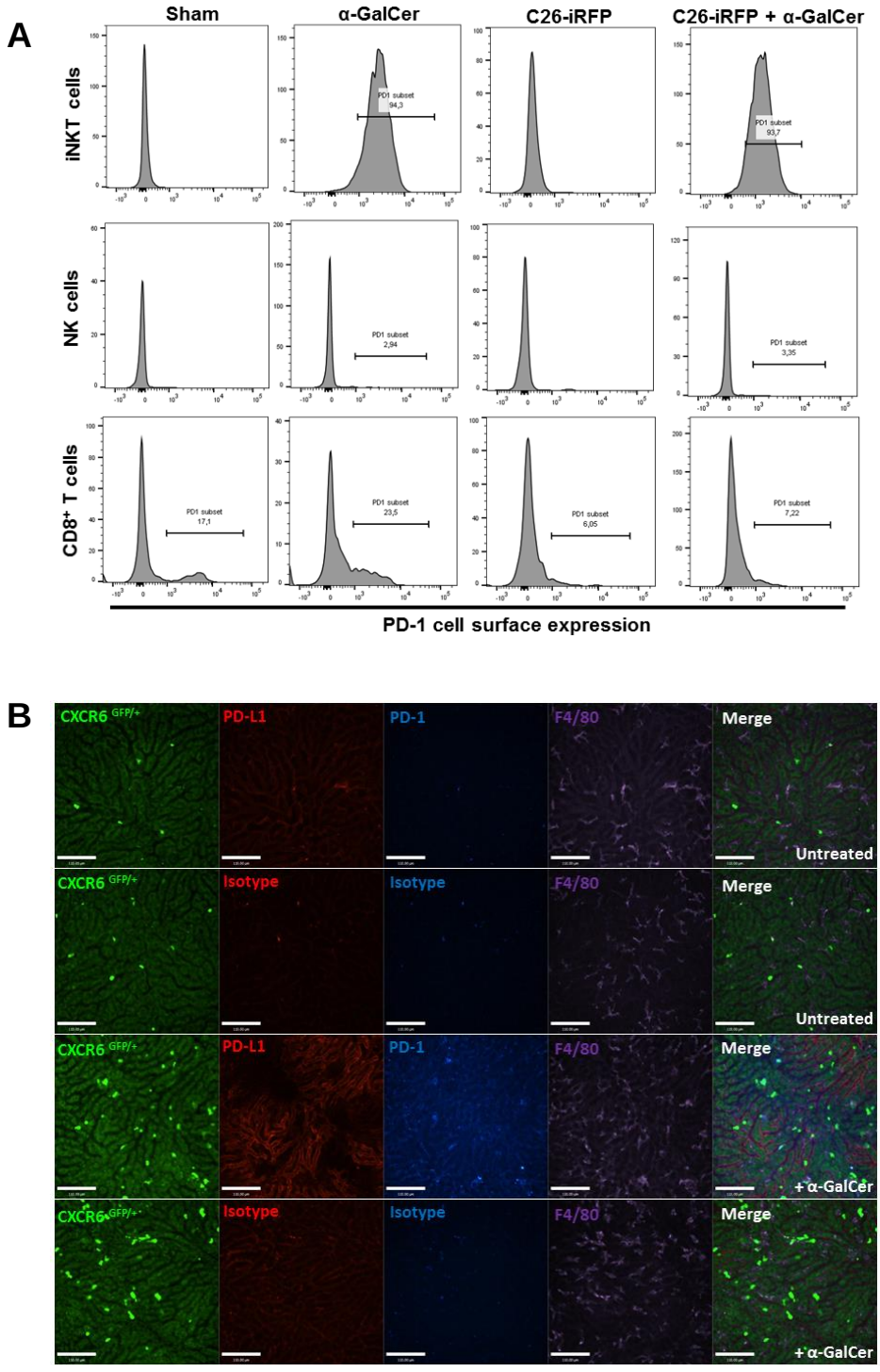


Figure S4. Upregulation of PD-1 and PD-L1 in the hepatic tumor micro-environment in response to α-GalCer immunotherapy and CD1d expression by CT26-iRFP cells. **A,** Representative histograms of PD-1 cell surface expression on iNKT cells, NK cells and CD8 T cells isolated from liver cell suspensions 5 days after the indicated treatments. **B,** Representative pictures of intravital liver image sequences of untreated and 24h after α-GalCer injected *CXCR6*$^{gfp/+}$ mice. Mice received either anti-PD-1 or anti-PD-L1 antibodies or, the respective isotype controls.

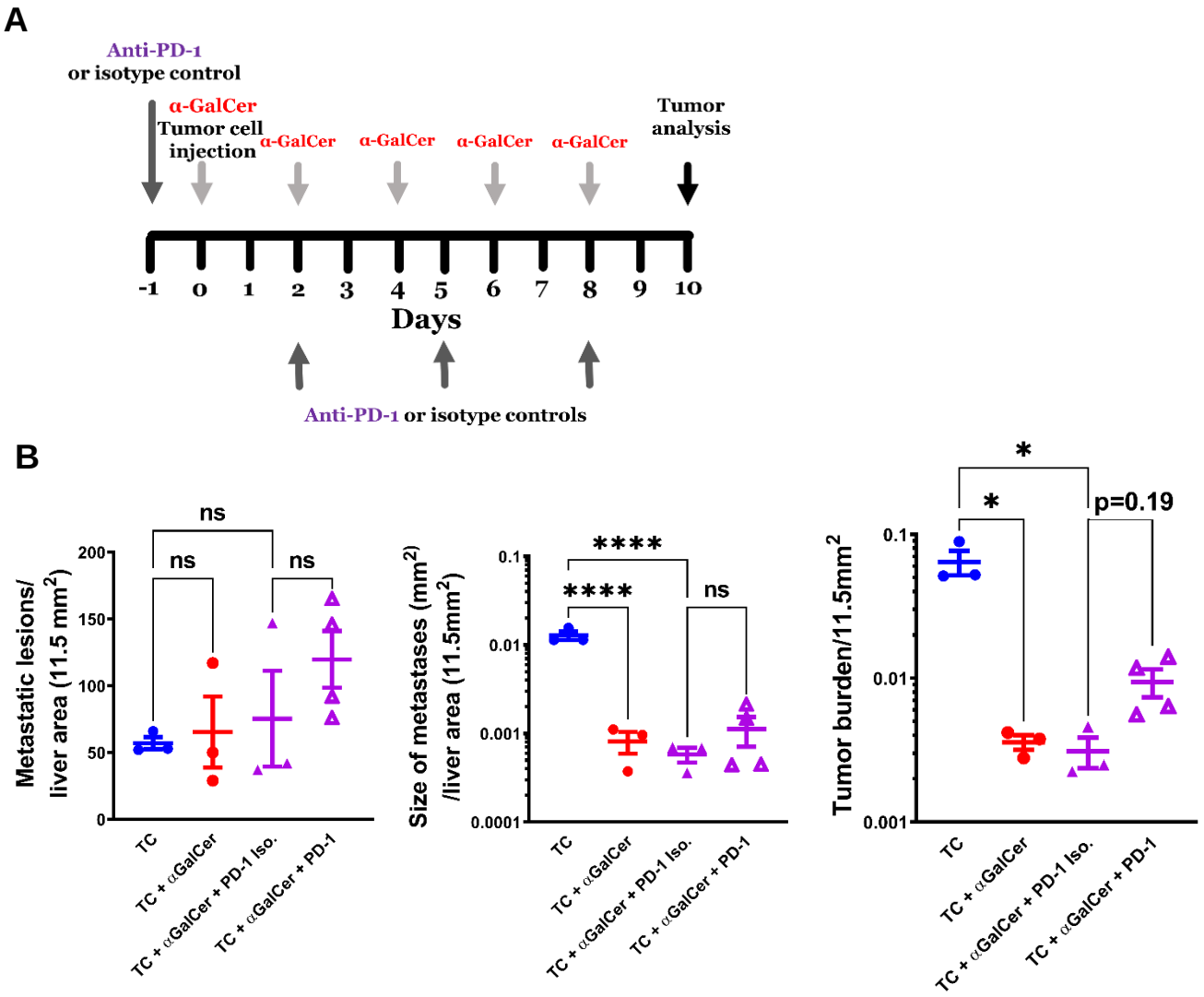
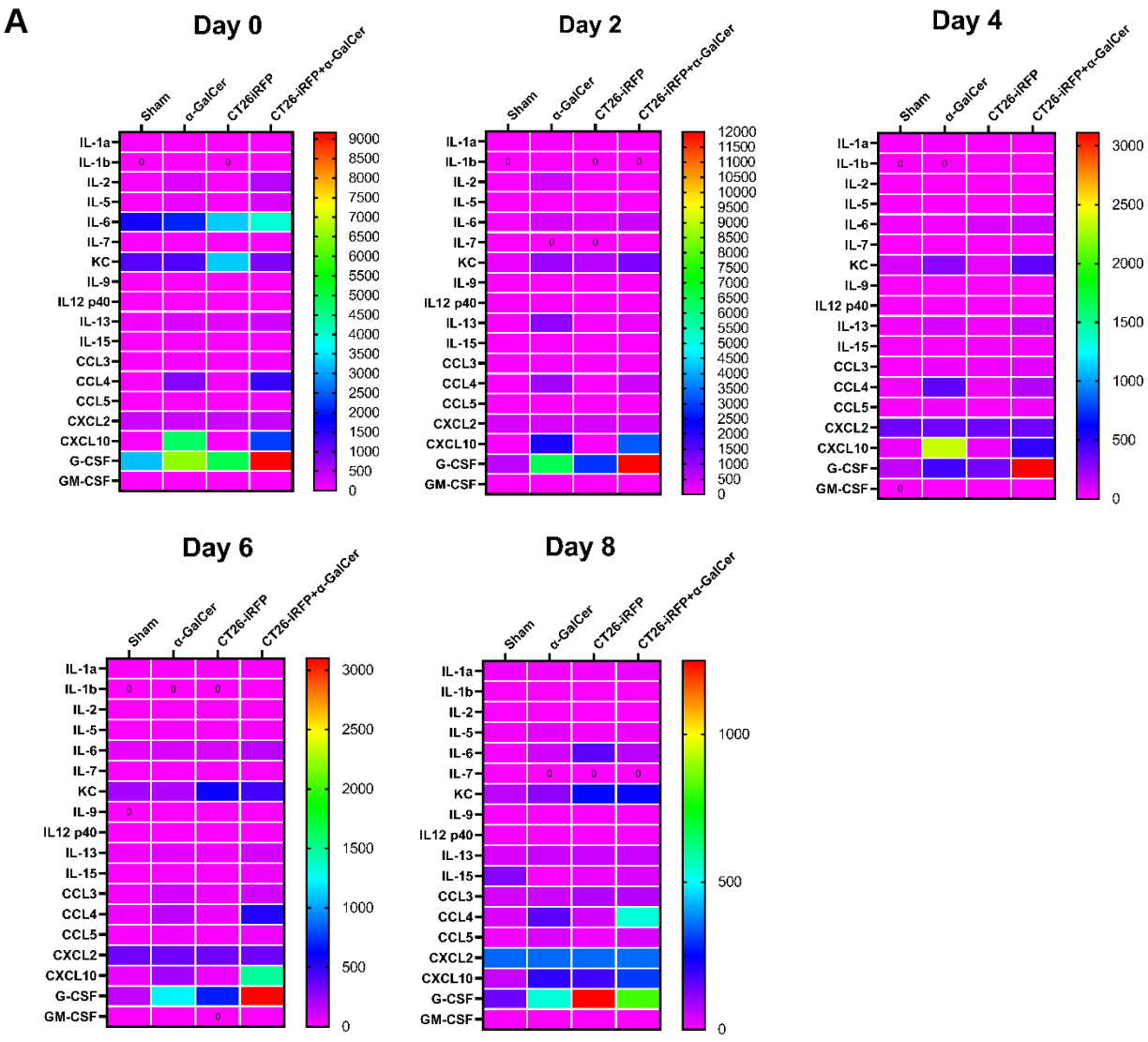


Figure S5. *In vivo* blocking of PD-1. **A,** Shows the timeline for the injection of anti-PD-1 and the respective isotype control in addition to α-GalCer treatment and the analysis of liver tumors. **B,** Shows the quantification of the liver metastases at day 10. TC= CT26-iRFP. Blue: CT26-iRFP (n=3 mice), red: CT26-iRFP+ α-GalCer (n=3 mice), purple triangle: CT26-iRFP+ α-GalCer + 2A3 isotype control (n=3 mice), open purple triangle: CT26-iRFP+ α-GalCer + anti-PD-1. Error bars show mean±s.e.m.; One-way Anova with Dunn's multiple comparisons test *p≤0.05 and ****p≤0.0001 indicate statistically significant differences.


A
Day 0
Day 2
Day 4
Day 6
Day 8
Sham
α-GalCer
CT26-iRFP
CT26-iRFP+α-GalCer
IL-1a
IL-1b
IL-2
IL-5
IL-6
IL-7
KC
IL-9
IL12 p40
IL-13
IL-15
CCL3
CCL4
CCL5
CXCL2
CXCL10
G-CSF
GM-CSF

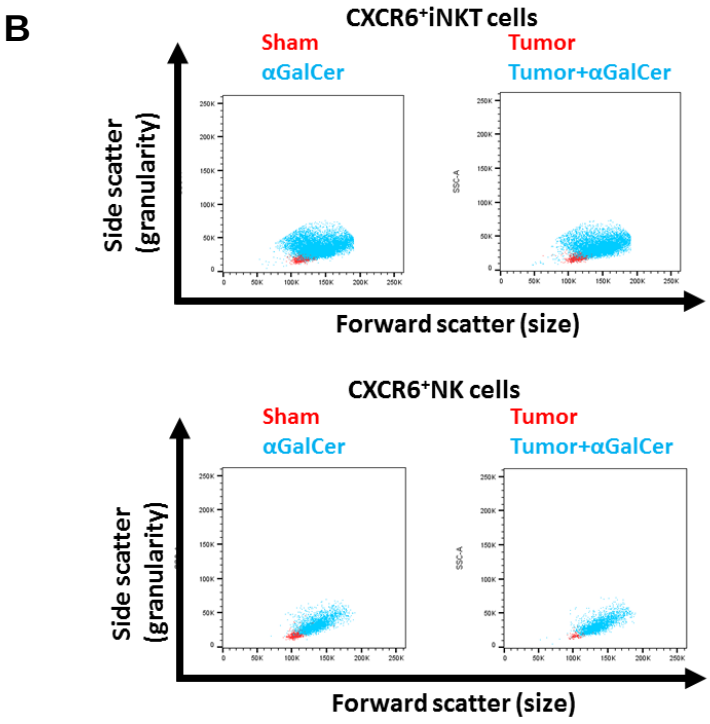

B
CXCR6⁺iNKT cells
Sham
αGalCer
Tumor
Tumor+αGalCer
Side scatter (granularity)
Forward scatter (size)
CXCR6⁺NK cells
Sham
αGalCer
Tumor
Tumor+αGalCer
Side scatter (granularity)
Forward scatter (size)

Figure S6, A, Heat maps representing additional plasma cytokines and chemokines measured in mice at different timepoints after treatment. Using graphpad prism a heat map was drawn based on the mean values of individual cytokine or chemokine concentrations (pg/ml) depicted in each row. The rainbow color scale ranges from the highest mean value (red) to the lowest mean value (purple). Columns show cytokines measured 4h after α-GalCer treatment at day 0, day 2, day 4, day 6 and day 8, n=3 for all treatment groups. **B,** Size and granularity of $CXCR6^+$ iNKT cells (top) and $CXCR6^+$ NK cells (bottom) are depicted at day3 after treatment by side and forward scatter in sham (splenectomised) and tumor (CT26-iRFP) bearing mice (red dot blots) and in α-GalCer treated and tumor (CT26-iRFP) + α-GalCer treated mice (blue dot blots). The dot blots represent one of four independent experiments.

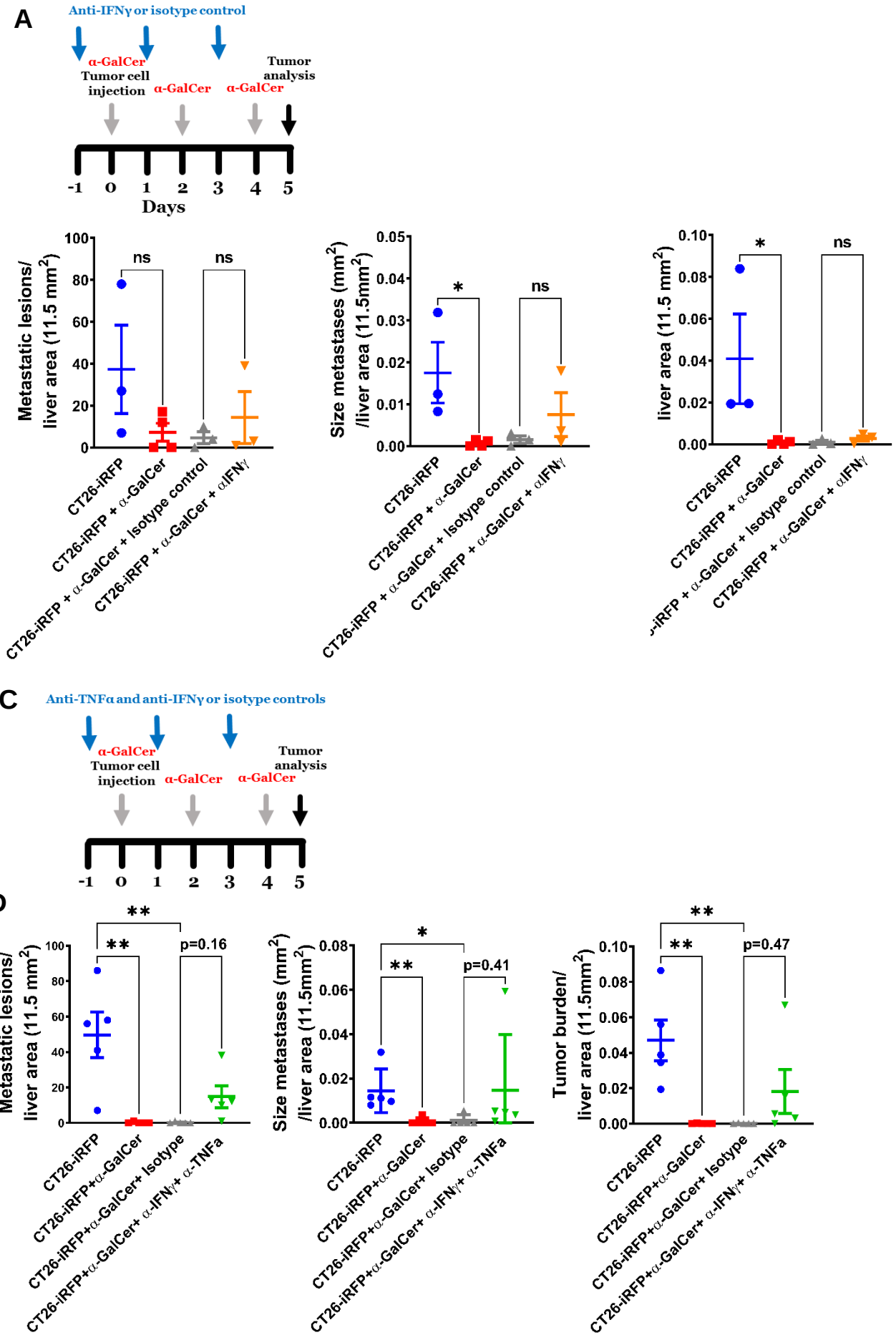
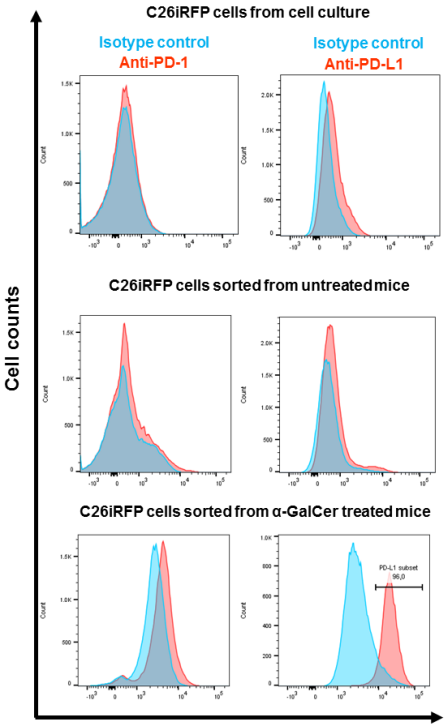

A
Anti-IFNγ or isotype control
α-GalCer
Tumor cell injection
α-GalCer
α-GalCer
Tumor analysis
-1 0 1 2 3 4 5
Days
B
Metastatic lesions/ liver area (11.5 mm2)
ns
ns
Size metastases (mm2) /liver area (11.5mm2)
*
ns
liver area (11.5 mm2)
*
ns
CT26-iRFP
CT26-iRFP + α-GalCer
CT26-iRFP + α-GalCer + Isotype control
CT26-iRFP + α-GalCer + αIFNγ
C
Anti-TNFα and anti-IFNγ or isotype controls
α-GalCer
Tumor cell injection
α-GalCer
α-GalCer
Tumor analysis
-1 0 1 2 3 4 5
D
Metastatic lesions/ liver area (11.5 mm2)
**
**
p=0.16
Size metastases (mm2) /liver area (11.5mm2)
*
**
p=0.41
Tumor burden/ liver area (11.5mm2)
**
**
p=0.47
CT26-iRFP
CT26-iRFP+α-GalCer
CT26-iRFP+α-GalCer+ Isotype
CT26-iRFP+α-GalCer+ α-IFNγ+ α-TNFa

Figure S7. A pro-inflammatory hepatic tumor micro-environment contributes to tumor control. **A,** Schematic of IFNγ neutralization and α-GalCer treatment in BALB/c wild type mice. **B,** Assessment of liver metastases after IFNγ depletion at day 5. CT26-iRFP n=3 mice, CT26-iRFP + α-GalCer n=4 mice, CT26-iRFP + α-GalCer +Isotype control n=3 mice, CT26-iRFP + α-GalCer + anti-IFNγ n=3 mice, error bars show mean±s.e.m.; One-way Anova, *p<0.05. **C,** Schematic of combined IFNγ + TNFα neutralization and α-GalCer treatment in BALB/c wild type mice. **D,** Quantification of liver metastases at day5 after treatment in BALB/c wild type mice. CT26-iRFP n=5 mice, CT26-iRFP + α-GalCer n=5 mice, CT26-iRFP + α-GalCer +Isotype control n=5 mice, CT26-iRFP + α-GalCer + anti-IFNγ+ anti-TNFα n=5mice. Error bars show mean±s.e.m.; One-way Anova with Dunn's multiple comparisons test, *p<0.05 and **p<0.01.

A


C26iRFP cells from cell culture
Isotype control
Anti-PD-1
Isotype control
Anti-PD-L1
C26iRFP cells sorted from untreated mice
C26iRFP cells sorted from α-GalCer treated mice
Cell counts
Count
PD-L1 subset
96,0

B

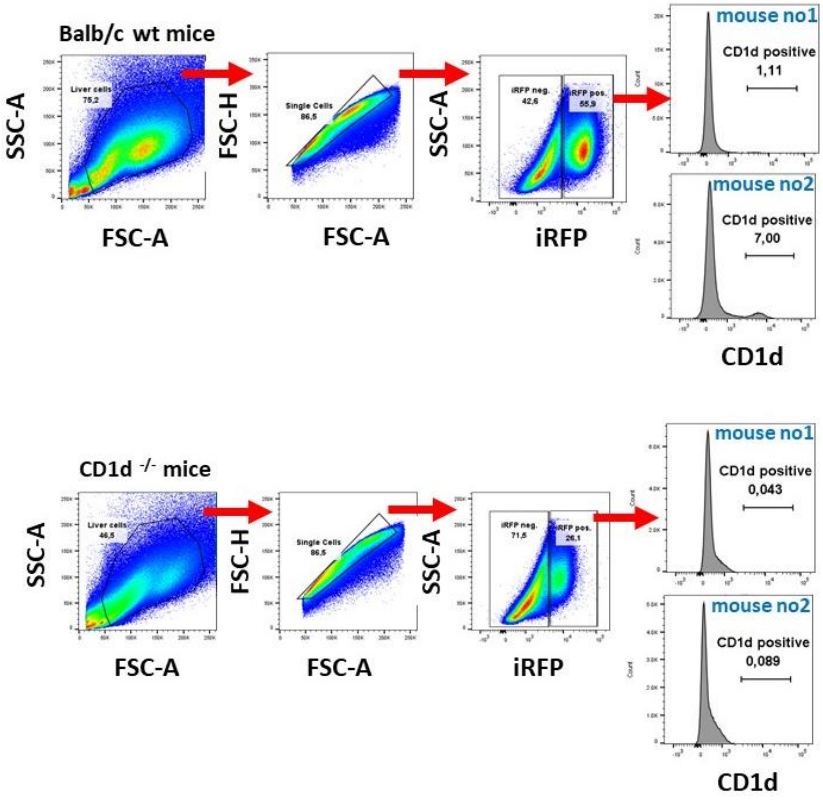

Balb/c wt mice
SSC-A
FSC-A
Liver cells
75,2
FSC-H
FSC-A
Single Cells
86,5
SSC-A
iRFP
iRFP neg.
42,6
iRFP pos.
55,9
mouse no1
CD1d positive
1,11
mouse no2
CD1d positive
7,00
CD1d
CD1d -/- mice
SSC-A
FSC-A
Liver cells
46,8
FSC-H
FSC-A
Single Cells
86,5
SSC-A
iRFP
iRFP neg.
71,5
iRFP pos.
26,1
mouse no1
CD1d positive
0,043
mouse no2
CD1d positive
0,089
CD1d

Figure S8. A, Flow cytometric assessment of PD-1 and PD-L1 cell surface expression on tumor cells isolated from cell culture (top row) and after sorting of iRFP-positive cancer cells at day 10 from untreated mice (middle row) and α-GalCer treated mice (bottom row). **B,** Shows the flow cytometric gating strategy to test for CD1d expression by CT26-iRFP cells in two Balb/c wild type (left) and two CD1d deficient mice (right) at day 10 after tumor cells were injected. SSC-A=side scatter area, FCS-A=forward scatter area, FCS-H=forward scatter height.

Supplementary Video captions:

Video S1. Liver intravital imaging of *CXCR6*$^{gfp/+}$ mice after splenectomy. iNKT cells are illustrated in bright green, Kupffer cells (anti-F4/80) in white and the vasculature (anti-CD31) in red. Dark green are auto-fluorescent hepatocytes. The 15 min long image sequence (20x lens) was recorded with 10 sec between frames. Scale bar: 90µm. The images were corrected for contrast. Elapsed time is shown at the top right.

Video S2. Liver intravital imaging of *CXCR6*$^{gfp/+}$ mice 24h after αGalCer treatment. iNKT cells are illustrated in bright green, Kupffer cells (anti-F4/80) in whit and the vasculature (anti-CD31) in red. Darker green are auto-fluorescent hepatocytes. The 10 min long image sequence (10x lens) was recorded with 9 sec between frames. Scale bar: 100µm. The images were corrected for contrast (Cy7 channel used for recording F4/80 cells). Elapsed time is shown at the top right.

Video S3 and S4. Behaviour of iNKT cells in the tumor micro-environment of the liver. iNKT cells (bright green) are visualized in *CXCR6*$^{gfp/+}$ mice, auto-fluorescent hepatocytes are shown in dark green. Colon carcinoma metastases (CT26-iRFP) are shown in blue at 3 days after inoculation. Crawling pattern of iNKT cells was recorded (10x lens) as 1h long image sequence with 15 sec between frames. Scale bar: 90µm. Images were corrected for contrast and brightness. Elapsed time is shown at the top right. **S3,** Liver of tumor bearing mice; **S4,** Liver after CT26iRFP injection and repeated α-GalCer administration.