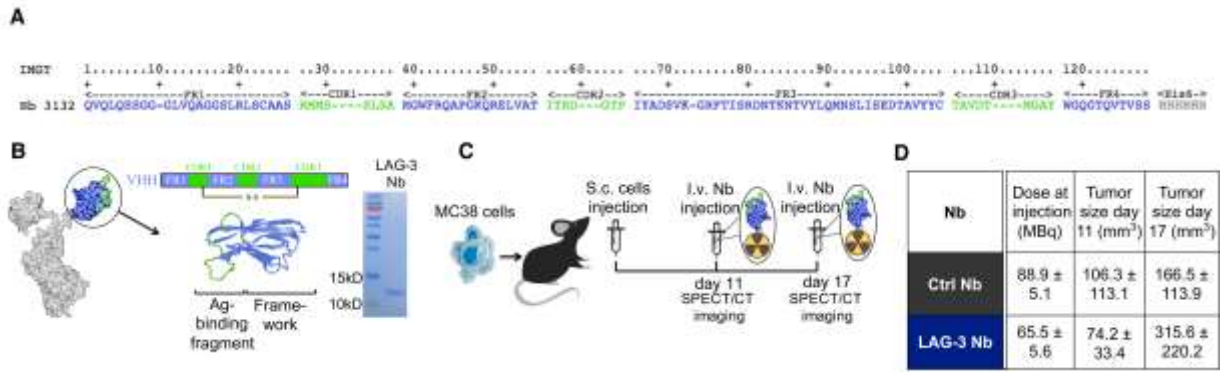
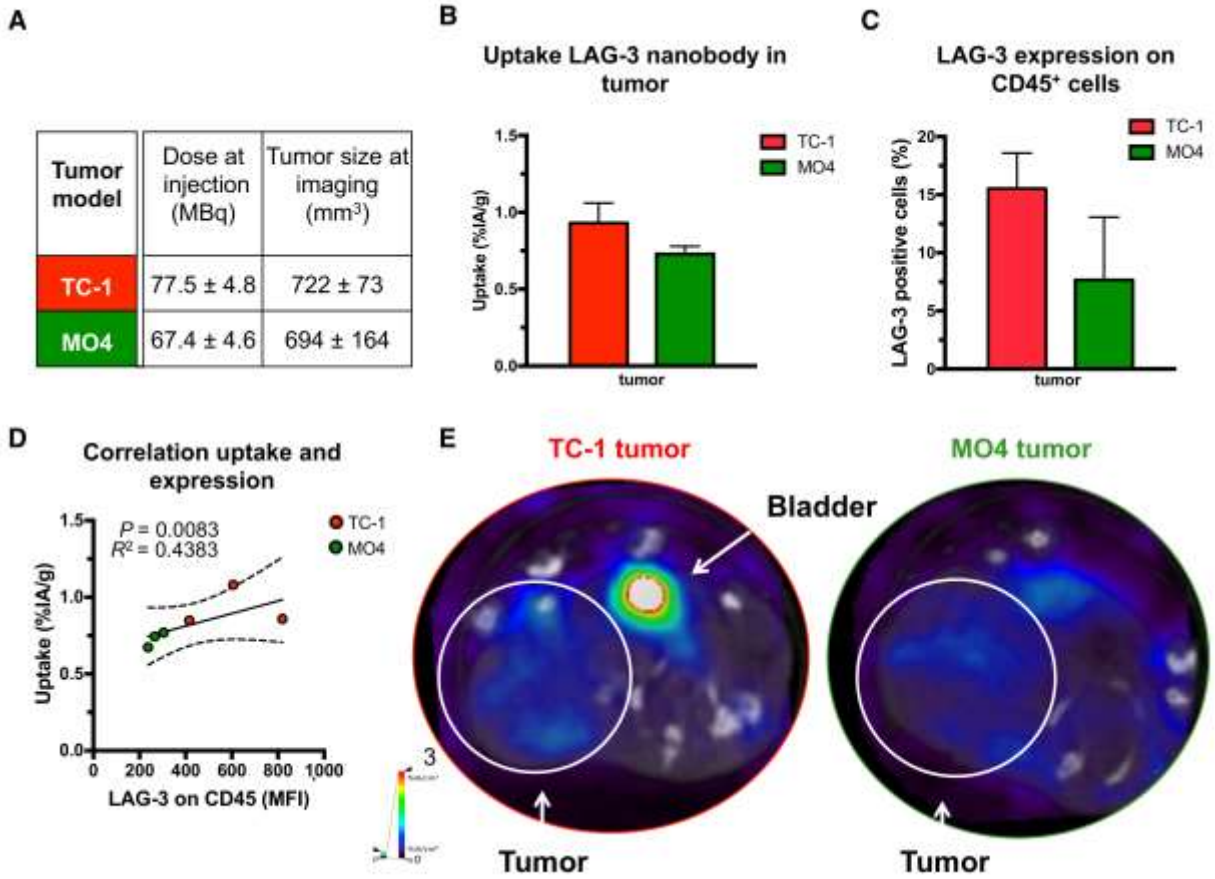


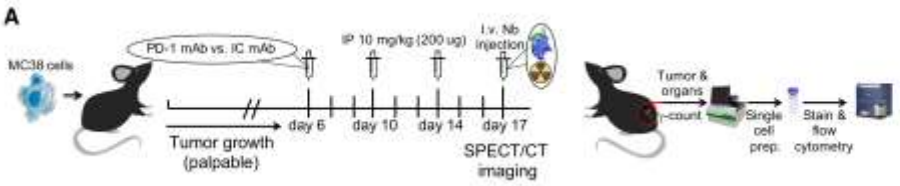
Nb	Dose at injection (MBq)	Tumor size day 11 (mm^3)	Tumor size day 17 (mm^3)
Ctrl Nb	88.9 ± 5.1	106.3 ± 113.1	166.5 ± 113.9
LAG-3 Nb	65.5 ± 5.6	74.2 ± 33.4	315.6 ± 220.2

Supplemental Figure 1: Injection of ^{99m}TC-labeled LAG-3 nanobody "3132" in MC38-bearing mice. (**a**) Amino acid sequence of the nanobody. (**b**) Structural model of a llama light-chain-deficient antibody from which the nanobody is derived and a Coomassie blue-stained protein gel loaded with 20µg of purified LAG-3 nanobody. (**c**) Timeline of the experiment. (**d**) Dose of injected radiolabeled LAG-3 nanobody (n = 18) or control nanobodies (n = 6) in MC38-bearing mice and the average tumor size at day of imaging.



Tumor model	Dose at injection (MBq)	Tumor size at imaging (mm^3)
TC-1	77.5 ± 4.8	722 ± 73
MO4	67.4 ± 4.6	694 ± 164

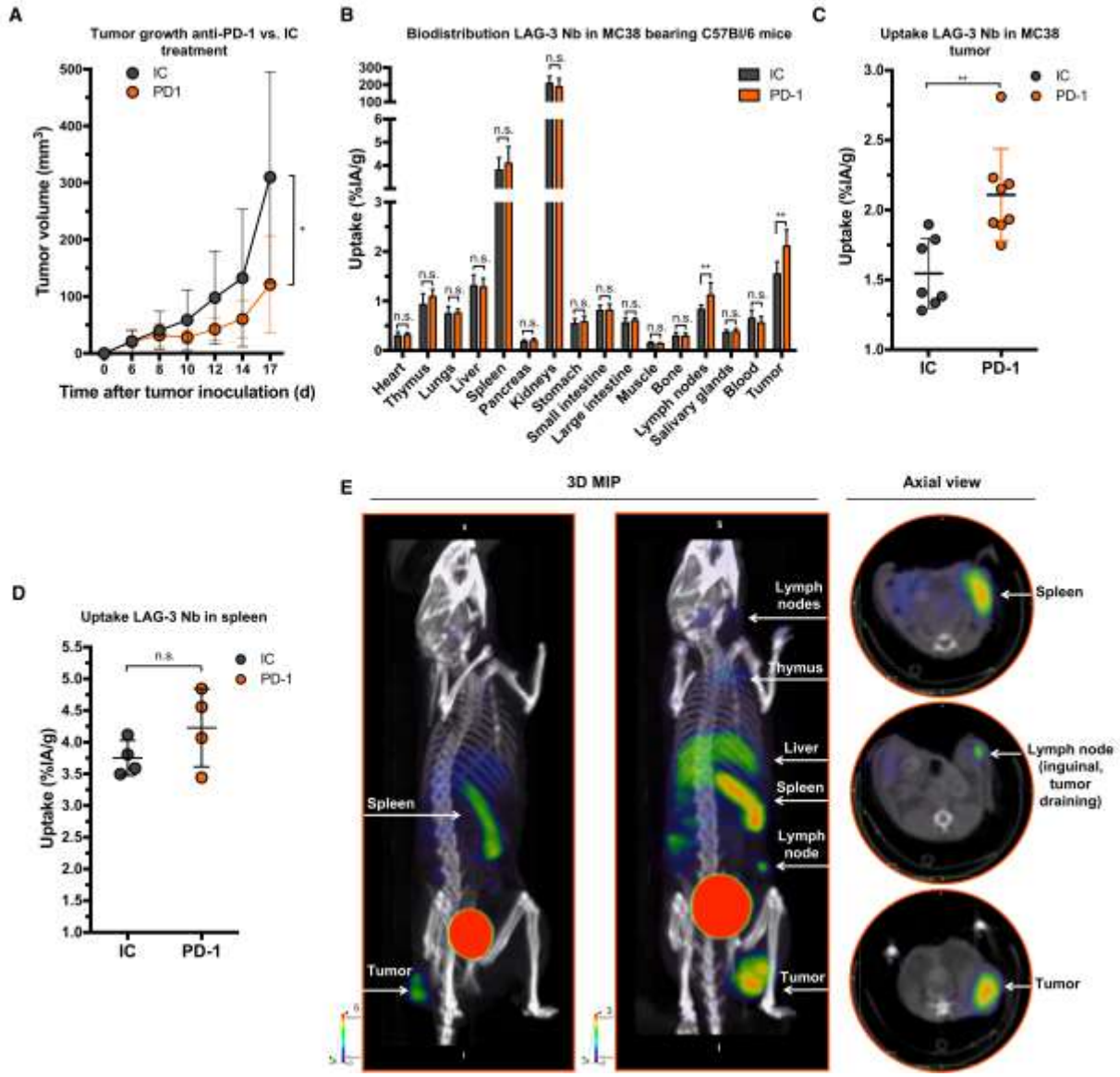
Supplemental Figure 2: SPECT/CT-imaging of LAG-3 using radiolabeled LAG-3 nanobody in TC-1 or MO4-bearing mice. (**a**) Dose of injected radiolabeled LAG-3 nanobody in mice bearing TC-1 (n=3) or MO4 (n=3) tumors and tumor volume at the time of imaging. (**b**) LAG-3 nanobody tracer tumor uptake levels as determined by *ex vivo* γ-counting of dissected tumors. (**c**) Percentage of LAG-3^{+}/CD45^{+} immune cells in tumors. (**d**) Correlation plot of LAG-3 expression (MFI) on immune cells (CD45^{+}) in TC-1 or MO4 tumors as analyzed by flow cytometry (x-axis) and the *ex vivo* γ-counting of the tumors (%IA/g, y-axis). (**e**) Representative axial SPECT/CT-images of TC-1 or MO4-bearing mice i.v. injected with ^{99m}Tc-labeled LAG-3 nanobody.



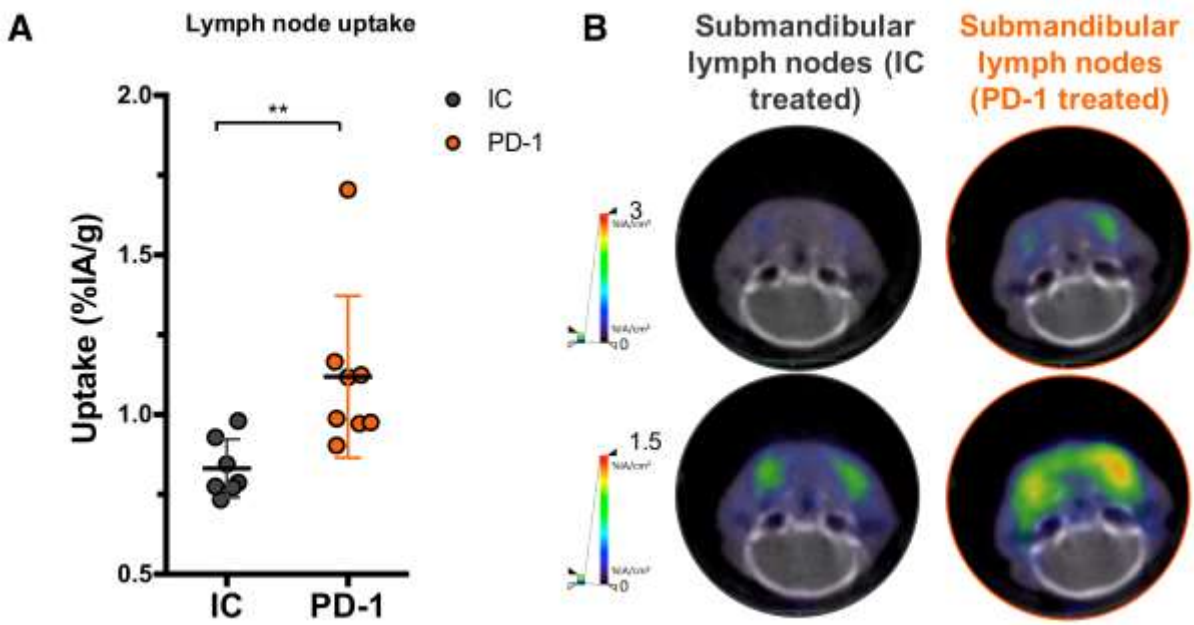
B

Condition	IC treated	PD-1 treated
Injected dose (MBq) **LAG-3 Nb**	75.7 ± 5.2	65.2 ± 3.3
Tumor size at imaging (mm^3)	310 ± 186	121 ± 86

Supplemental Figure 3: Evaluating PD-1-blockade versus IC in MC38-bearing mice. (**a**) Timeline of the experiment. (**b**) Tumor size at time of imaging and dose of injected radiolabeled LAG-3 nanobody in MC38-bearing mice treated with either IC-mAbs (n=7) or PD-1-blocking mAbs (n=8).



Supplemental Figure 4: Effect of PD-1-blockade versus IC in MC38-bearing mice on LAG-3 detection in the tumor and spleen. (**a**) Growth kinetics of MC38-tumors treated with IC-mAbs (n=7) or PD-1-blocking mAbs (n=8). (**b**) E*x vivo* γ-counting of isolated organs from MC38-bearing mice, treated with anti-PD-1 or IC, 80 minutes after injection of LAG-3 nanobody tracer. (**c**) Uptake levels of LAG-3 nanobody tracer in MC38-tumors of anti-PD-1 or IC-treated mice, as determined by *ex vivo* γ-counting. (**d**) Uptake levels of LAG-3 nanobody tracer in spleens of anti-PD-1 or IC-treated mice, as determined by *ex vivo* γ-counting. (**e**) SPECT/CT-imaging of anti-PD-1 treated mice i.v. injected with LAG-3 nanobody tracer, scaled at 0-5 (left) or 0-3 %IA/cm^3 (right), showing specific signals in the spleen, tumor and tumor-draining lymph node (n=2). Note the removal of both kidneys before image acquisition.



Supplemental Figure 5: Effect of PD-1-blockade versus IC in MC38-bearing mice on LAG-3 detection in the submandibular lymph nodes. (**a**) Uptake levels of LAG-3 nanobody tracer in submandibular lymph nodes of anti-PD-1 or IC-mAb treated mice, as determined by *ex vivo* γ-counting. (**b**) Representative axial SPECT/CT-images of submandibular lymph nodes of anti-PD-1 or IC-mAb treated mice i.v. injected with ^{99m}Tc-labeled LAG-3, scaled at 0-3 %IA/cm^3 (above) and 0-1.5 %IA/cm^3 (below).