

## Supplementary data for

### **Elevated target expression by dual PD-L1 and 4-1BB engagement is associated with <sup>89</sup>Zr-PD-L1x4-1BB bispecific Mabcalin tumor uptake**

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Table S2. In vivo tumor-to-blood ratio (SUVmax/SUVmean) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 1, 2 and 4 days post-injection.

Table S3. Absolute ex vivo uptake (%ID/g) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 7 days post-injection in lymphoid tissues and the tumor.

Table S4. Ex vivo uptake (tissue-to-blood AUC<sub>WB, 0-7 days</sub>) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 7 days post-injection in lymphoid tissues and the tumor.

Table S5. Absolute ex vivo uptake (%ID/g) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 4 days post-injection in lymphoid tissues and the tumor.

Table S6. Ex vivo uptake (tissue-to-blood  $AUC_{WB, 0-4 \text{ days}}$ ) of  $^{89}\text{Zr}$ -mPD-L1x4-1BB and  $^{89}\text{Zr}$ -hPD-L1x4-1BB in h4-1BB KI or WT mice at 4 days post-injection in lymphoid tissues and the tumor.

Table S7. Ex vivo uptake (tissue-to-blood  $AUC_{WB, 0-7 \text{ days}}$ ) of  $^{89}\text{Zr}$ -mPD-L1x4-1BB and  $^{89}\text{Zr}$ -hPD-L1x4-1BB in h4-1BB KI or WT mice at 7 days post-injection in non-lymphoid tissues.

Table S8. Ex vivo uptake (tissue-to-blood  $AUC_{WB, 0-7 \text{ days}}$ ) of  $^{89}\text{Zr}$ -mPD-L1x4-1BB and  $^{89}\text{Zr}$ -hPD-L1x4-1BB in h4-1BB KI or WT mice at 4 days post-injection in non-lymphoid tissues.

## Supplemental Methods

### Conjugation and <sup>89</sup>Zr-labeling of mPD-L1xh4-1BB and hPD-L1xh4-1BB

mPD-L1xh4-1BB and hPD-L1xh4-1BB Mabcalins (Servier and Pieris Pharmaceuticals) were conjugated after a buffer exchange to 25 mM NaHCO<sub>3</sub> in 0.9% NaCl pH 9.0 with a PD-10 desalting column (Cytiva, #28918007), followed by 60 min incubation with p-SCN-Bn-deferoxamine (DFO; Macrocyclus, #B-705) at 20 °C. The DFO-conjugated Mabcalins were purified using a PD-10 desalting column and formulated in 50 mM histidine (Sigma-Aldrich), 50 mM NaCl (Sigma-Aldrich), 200 mM arginine (Sigma-Aldrich), 0.01% polysorbate 80 (Fisher Scientific), pH 6.5 and stored at -80 °C DFO-PD-L1x4-1BB Mabcalins were radiolabeled with <sup>89</sup>Zr, as described previously [1, 2]. Potential aggregation and fragmentation of the conjugated and radiolabeled PD-L1x4-1BB were assessed with size exclusion ultra-high performance liquid chromatography equipped with BioSep or Yarra gel filtration SEC-s3000 column (Phenomenex, # 00H-2146-K0, # 00H-4513-K0), as previously described (Figure S3C-F) [3]. Radiochemical purity was measured by trichloroacetic acid precipitation assay [4].

The potential targets for DFO-conjugation to surface-exposed lysine side chains of the hPD-L1xh4-1BB were determined by homology modeling with Molecular Operating Environment (MOE) 2020.09 (Chemical Computing Group ULC, Montreal, QC, Canada). The Anticalin protein moiety targeting 4-1BB was homology modeled based on the protein data bank templates 1DFV. The fragment antigen binding region of the hPD-L1 antibody building block was modeled using the antibody modeler function of MOE with the following template structures for the variable light chain: PDB ID.chain for frameworks, CDRs 1, 2, 3: 4R7D.H, 1YY9.C, 4YXL.L, 3HFM.L, and for the variable heavy chain: 4R7D.G, 2GK0.H,

2GK0.H, 1SBS.H. This modeling revealed two surface-exposed lysine residues close to the complementarity-determining regions targeting PD-L1 and seven surface-exposed lysine residues in the 4-1BB targeting binding regions of the Anticalin portion, respectively (Figure S3A-B). Therefore, multiple DFO to Mabcalin ratios were investigated to determine the effect of DFO-conjugation on binding affinities.

To evaluate the binding properties of DFO-PD-L1x4-1BB for 4-1BB and PD-L1, the relative potency, expressed as half-effective concentration (EC<sub>50</sub>) of DFO-Mabcalin divided by EC<sub>50</sub> of their respective unconjugated Mabcalin, was determined with an indirect enzyme-linked immunosorbent assay (ELISA) as described before [5]. The PD-L1 and 4-1BB extracellular domains (R&D Systems, #9049-B7-100, #9220-4B-100) were used as a target. DFO-conjugation did not affect PD-L1 binding, while 4-1BB binding was already impaired at a 1:2 ratio of PD-L1x4-1BB Mabcalin to DFO. Therefore, the lowest possible Mabcalin to DFO ratio of 1:2 needed for <sup>89</sup>Zr labeling at 30 – 167 MBq/mg with ≥ 95% radiochemical purity was considered optimal (Figure S3C-L). <sup>89</sup>Zr radiolabeling of DFO-hPD-L1x4-1BB did not affect target binding based on a radioactive ELISA (Figure S3K, L) [6]. DFO-hPD-L1x4-1BB can activate T-cells to the same degree as unmodified Mabcalin based on a staphylococcal enterotoxin B functional assay (Figure S3M) [7].

#### **Pharmacokinetics of DFO-hPD-L1x4-1BB in CD1 mice**

The effect of DFO-conjugation on the PK profile of hPD-L1x4-1BB was tested in male CD1 mice (Charles River Laboratories). The National Committee for Ethical Reflection on Animal Experimentation in France approved the study. hPD-L1x4-1BB or DFO-hPD-L1x4-1BB 10 mg/mL was administered intravenously in the caudal vein (n = 6 mice per group, 9 weeks of age). The mice were divided into two groups (n = 3 per time point), and micro-blood sampling was performed at 5 min, 4, 24, and 96 h or 1, 8, 48, and

168 h. The (DFO-)hPD-L1xh4-1BB plasma concentrations were determined with a MesoScale Discovery assay. A 384-well multi-array plate was coated overnight at 4°C with hPD-L1 at 1 µg/mL. After blocking with 2% bovine serum albumin and 0.1% polysorbate 20 in PBS (Gibco, pH 7.4), the collected plasma was incubated for 1 h at room temperature. (DFO-)hPD-L1xh4-1BB was detected with 1 µg/mL anti-NGAL Ab (clone PL713, BioGenes), anti-rabbit IgG Sulfo-Tag (Meso Scale Discovery; #R32AB-1), and 2x MSD Read Buffer (Meso Scale Discovery, #R92TC-1). The plates were measured using Sector Imager S600. The area under the plasma concentration-time curve over 168 h, half-life, and clearance were calculated with non-compartmental PK analysis with an in-house macro in Microsoft Excel software version office 365 (Servier; Figure S3N, O).

### **Plasma tracer integrity and autoradiography of tissues**

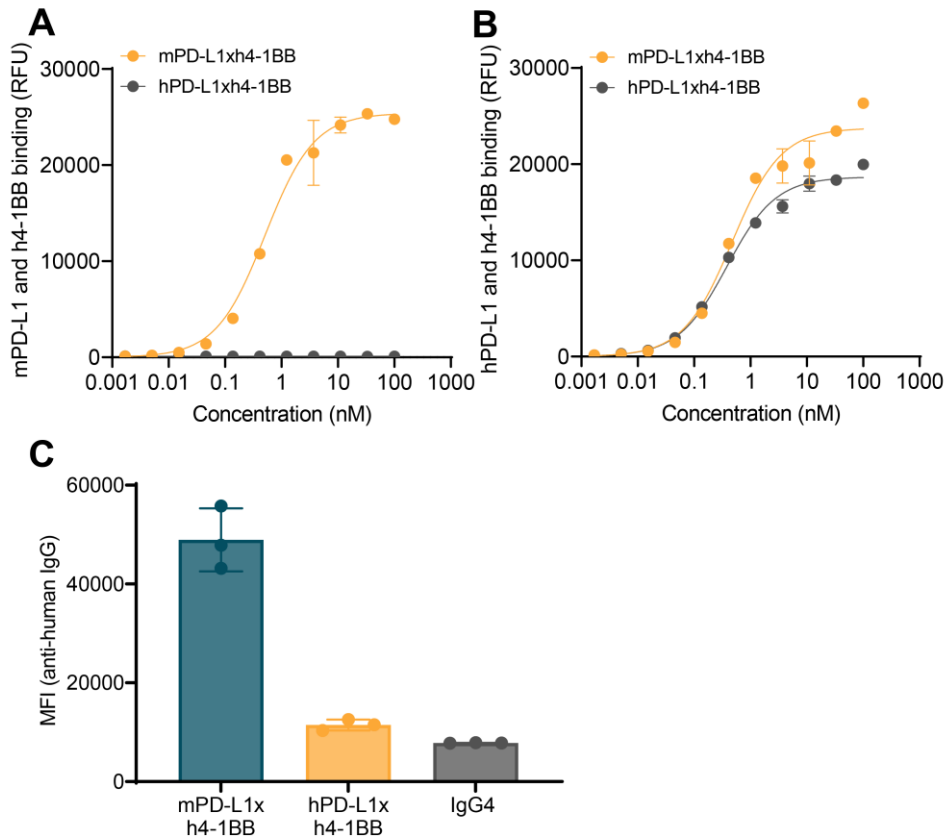
Plasma tracer integrity of <sup>89</sup>Zr-mPD-L1xh4-1BB and <sup>89</sup>Zr-hPD-L1xh4-1BB were confirmed with SDS-PAGE (Figure 3E). A 10% polyacrylamide gel (Bio-Rad; #4561034) was loaded with 250 counts/min of the plasma samples. The positive control, the injected tracer, stored at 4°C in 0.5% human serum albumin (Sanquin), was loaded within the same activity range. The gel was exposed overnight to an imaging plate (BAS-MS; Fujifilm) and read with a Typhoon laser scanner (Amersham). Autoradiography on 4 µm tumor and spleen sections was performed similarly. The tissue slides were exposed with 1.5 µL of 1%, 0.1%, and 0.01% injected tracer on silica gel on thin-layer chromatography Al foils (Sigma-Aldrich). The maximum gray value uptake in tissue slides was converted into %ID per area based on the diluted tracer standards using ImageJ version 1.53k. The autoradiography slides were subsequently stained with hematoxylin and eosin to analyze tissue viability and morphology.

## Supplemental References

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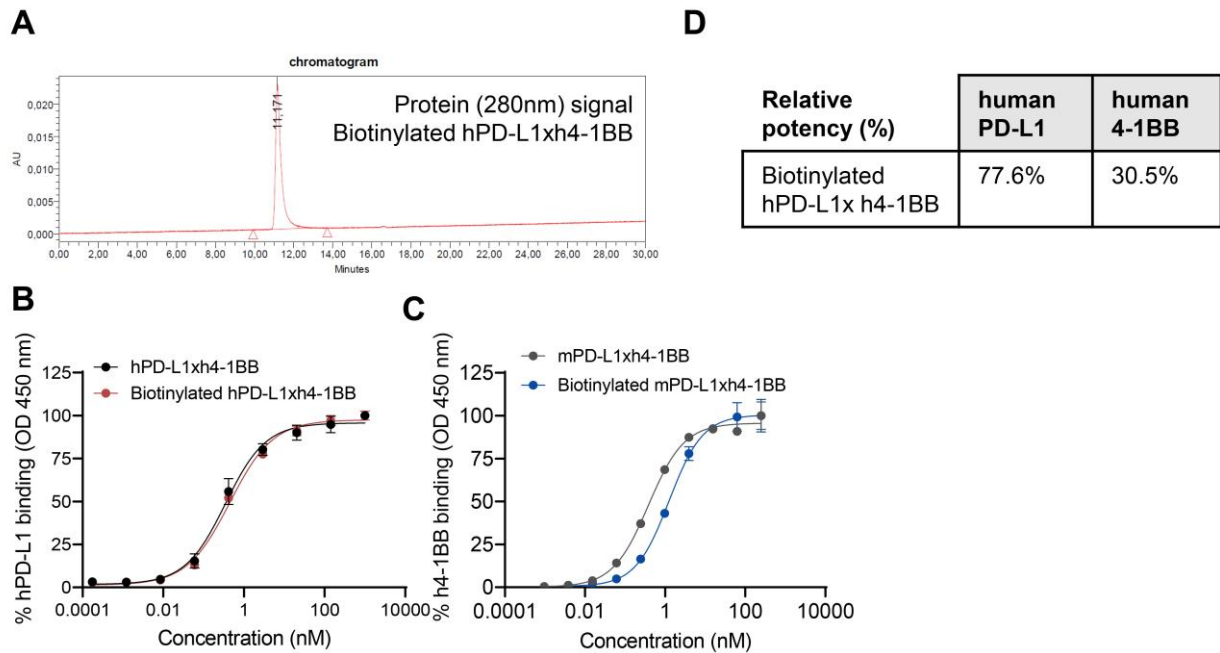
## Supplemental Figures



**Figure S1. Characterization of mPD-L1xh4-1BB and hPD-L1xh4-1BB.**

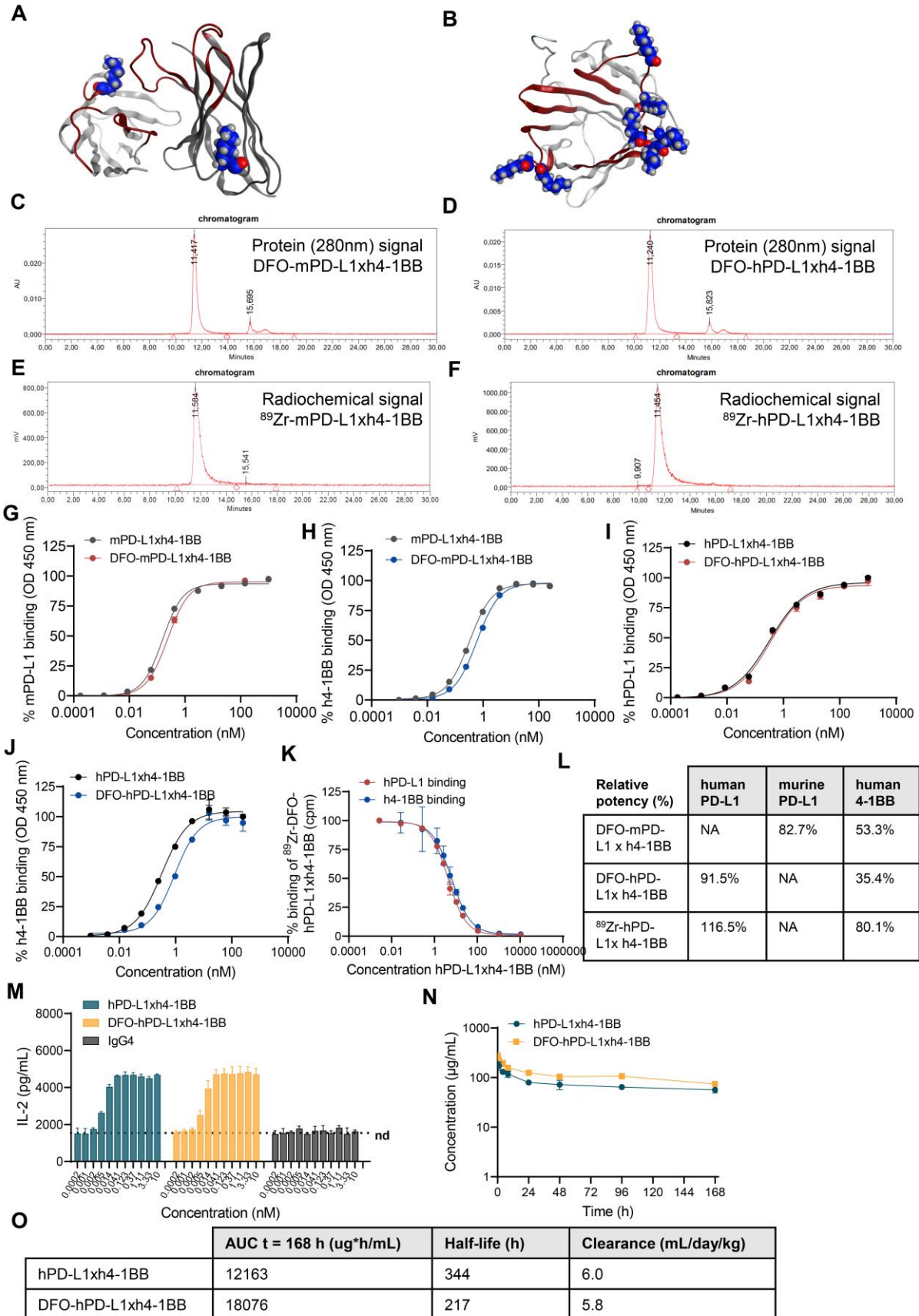
**(A)** Dual binding of mPD-L1xh4-1BB and hPD-L1xh4-1BB to recombinant murine PD-L1 and human 4-1BB assessed with ELISA. **(B)** Dual binding of mPD-L1xh4-1BB (cross-reactive to hPD-L1) and hPD-L1xh4-1BB to recombinant human PD-L1 and 4-1BB assessed with ELISA. **(C)** Binding of mPD-L1xh4-1BB, hPD-L1xh4-1BB, and IgG4 to murine PD-L1 positive MC38 tumor cells assessed with flow cytometry. Detection of antibodies was done with APC-anti-IgG (Jackson ImmunoResearch; 109-136-088). Data are presented as mean  $\pm$  standard deviation

APC: allophycocyanin; ELISA: enzyme-linked immunosorbent assay; h: human; m: murine; MC38: murine colon adenocarcinoma cell line; MFI: mean fluorescent intensity; nM: nanomolar; RFU: relative fluorescent units.



**Figure S2. Characterization of biotinylated hPD-L1xh4-1BB.**

(A) Size-exclusion ultra-high performance liquid chromatogram of the UV absorption at 280 nm of biotinylated hPD-L1xh4-1BB. (B) Binding of (biotinylated) hPD-L1xh4-1BB to recombinant human PD-L1 assessed by ELISA (n=3). (C) Binding of (biotinylated) hPD-L1xh4-1BB to recombinant human 4-1BB assessed by ELISA (n=3). In both ELISA experiments, the maximum specific concentration was similar between biotinylated and unconjugated Mabcalin. (D) Relative potencies of data presented in B and C.

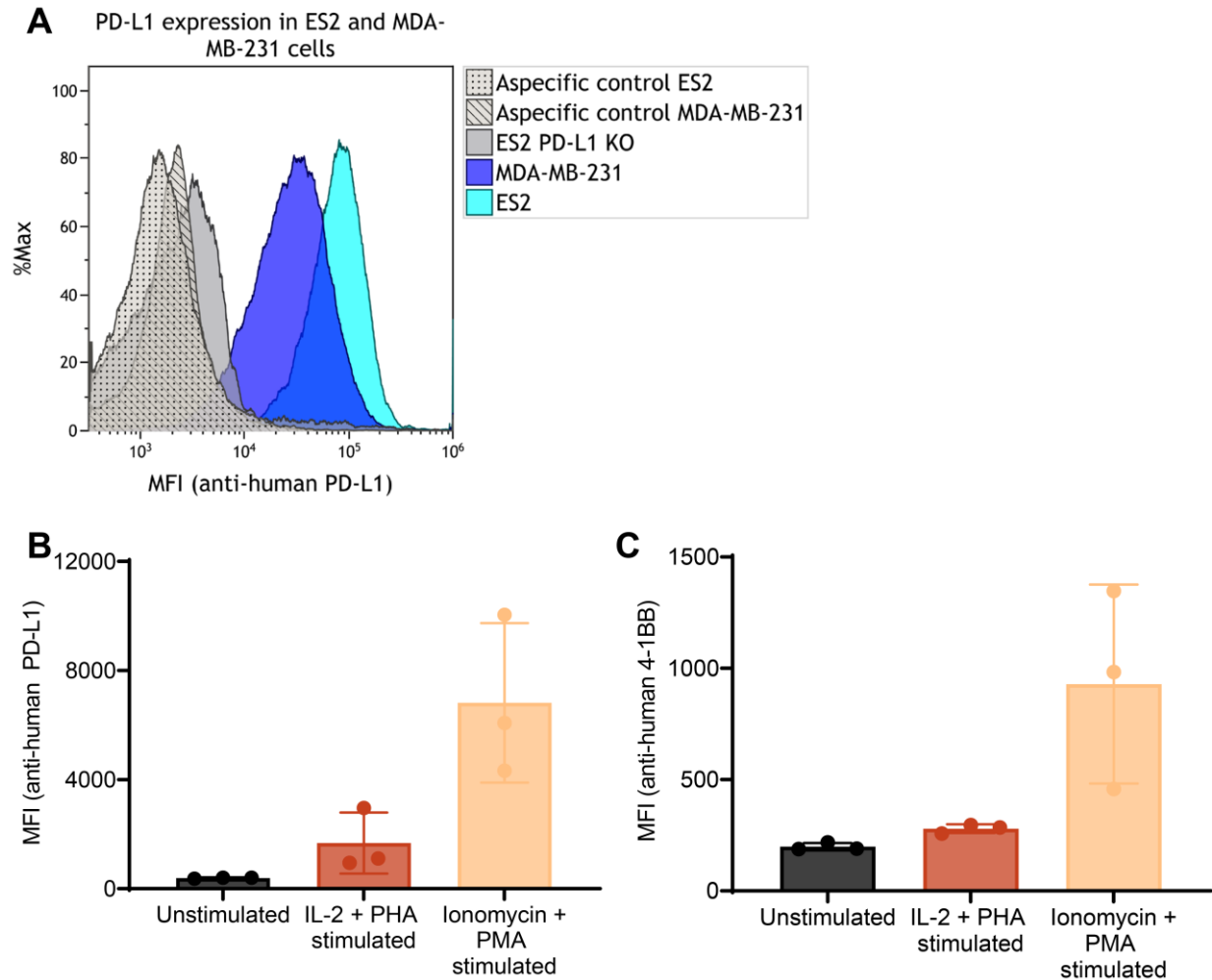


**Figure S3. Characterization of DFO-conjugated and <sup>89</sup>Zr-radiolabeled mPD-L1xh4-1BB and hPD-L1xh4-1BB.**

**(A)** Size-exclusion ultra-high performance liquid chromatogram of the UV absorption at 280 nm of biotinylated hPD-L1xh4-1BB. **(B)** Cartoon representation of the variable domain homology model of the hPD-L1xh4-1BB binding antibody portion of the Mabcalin. Complementarity-determining regions are shown in red, and constant regions of the variable fragment heavy and light chains are shown in dark and light grey, respectively. Surface-exposed lysine residues in the variable domain are shown as spheres with blue carbon atoms. **(C)** Cartoon representation of the Anticalin homology model of hPD-L1xh4-1BB. The four flexible regions of the Anticalin moiety involved in 4-1BB binding are shown in red, whereas the rest is in gray. Surface-exposed lysine residues are highlighted as in B. **(D)** Size-exclusion ultra-high performance liquid chromatogram of the UV absorption at 280 nm of DFO-mPD-L1xh4-1BB. **(E)** Size-exclusion ultra-high performance liquid chromatogram of the UV absorption at 280 nm of DFO-hPD-L1xh4-1BB. **(F)** Size-exclusion ultra-high performance liquid chromatogram of the <sup>89</sup>Zr-mPD-L1xh4-1BB radiochemical signal. **(G)** Size-exclusion ultra-high performance liquid chromatogram of the radiochemical signal of <sup>89</sup>Zr-hPD-L1xh4-1BB. **(H)** Binding of (DFO-) mPD-L1xh4-1BB to recombinant murine PD-L1 assessed by ELISA. **(I)** Binding of (DFO-) mPD-L1xh4-1BB to recombinant human 4-1BB assessed by ELISA. **(J)** Binding of (DFO-) hPD-L1xh4-1BB to recombinant human PD-L1 assessed by ELISA. Data from 3 individual experiments is presented with n = 3 per experiment. **(K)** Binding of (DFO-) hPD-L1xh4-1BB to recombinant human 4-1BB assessed by ELISA. Data from 3 individual experiments is presented with n=3 per experiment. In all ELISA experiments, the maximum specific

concentration was similar between DFO-conjugated and unconjugated Mabcalin. **(L)** Competitive binding affinity of  $^{89}\text{Zr}$ -hPD-L1xh4-1BB compared to unmodified hPD-L1xh4-1BB. Data from 3 individual experiments is presented with  $n = 2$  per experiment. **(M)** Relative potencies of data presented in H-K. **(N)** T-cell activation of peripheral blood mononuclear cells (PBMCs) measured as IL-2 secretion by staphylococcal enterotoxin B functional assay. The dashed line indicates IL-2 secretion of staphylococcal enterotoxin B-activated PBMCs without presented constructs. The result of one of the four tested donors is shown. **(O)** Plasma concentration-time profile following single intravenous administration of 10 mg/kg antibody into CD1 mice. Three mice per time point per group. **(P)** The area under the curve, half-life, and clearance of hPD-L1xh4-1BB and DFO-hPD-L1xh4-1BB in CD1 mice. Data are presented as mean  $\pm$  standard deviation.

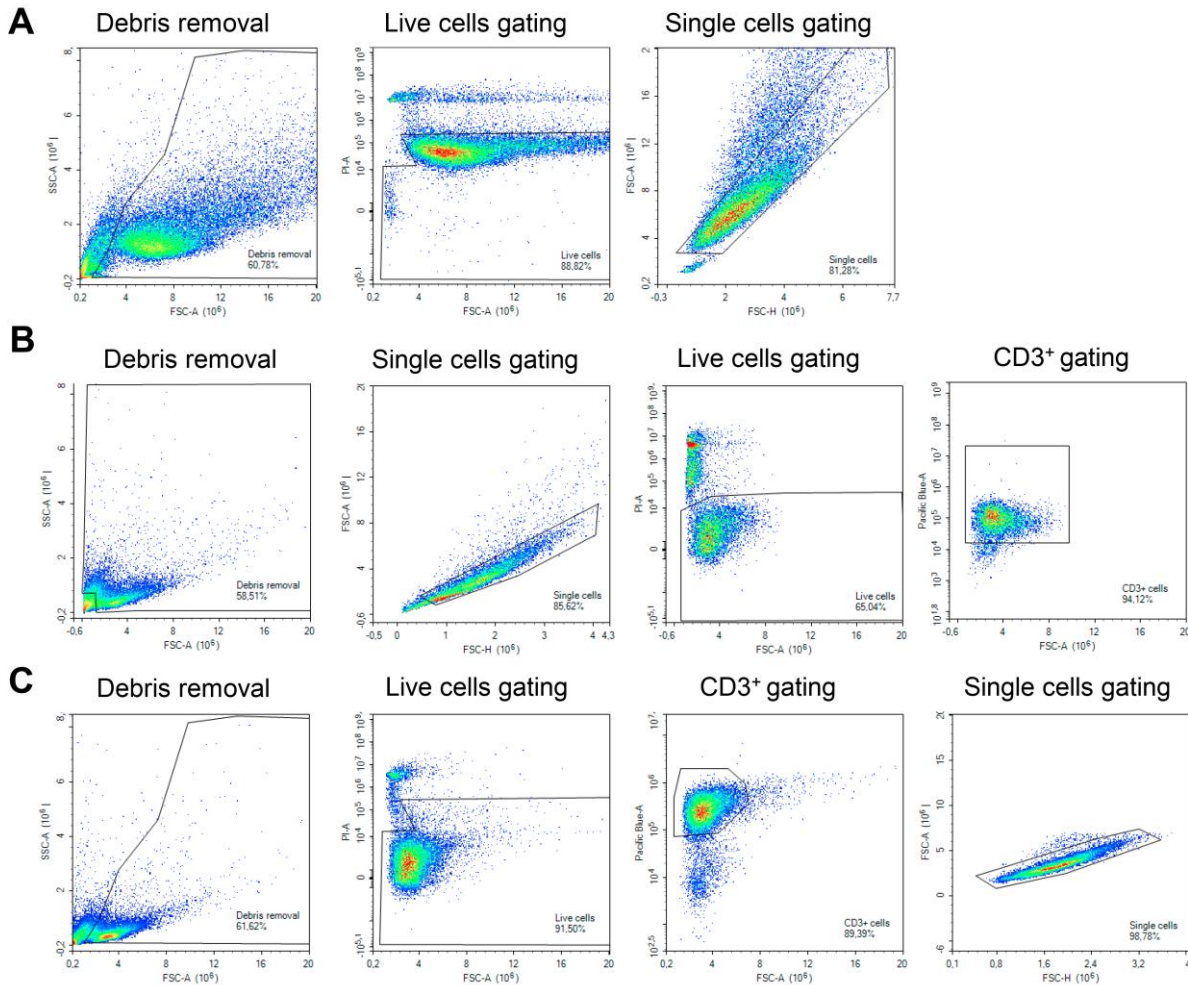
AUC: area under the curve; cpm: counts per min; h: human; IL-2: interleukin-2; m: murine; mV: microvolt; NA: not applicable; nd: not detected; nM: nanomolar; nm: nanometer; OD: optical density.



**Figure S4. Receptor expression levels on MDA-MB-231, ES2, ionomycin + PMA, and IL-2 + PHA stimulated human T-cells.**

**(A)** PD-L1 expression on MDA-MB-231, ES2 and ES2 PD-L1 knock-out cell lines. **(B)** PD-L1 expression of human T-cells of three individual PBMC donors after ionomycin + PMA activation for 16 h and IL-2 + PHA stimulation for 3 days. **(C)** 4-1BB expression of human T-cells of three individual PBMC donors after ionomycin/PMA stimulation for 16 h and IL-2/PHA activation for 3 days. Data are presented as mean  $\pm$  standard deviation.

ES2 PD-L1 KO: PD-L1 knock-out cell line; IL-2: interleukin-2; MFI: mean fluorescent intensity; PBMCs: peripheral blood mononuclear cells; PHA: lectin from Phaseolus vulgaris; PMA: phorbol myristate acetate.

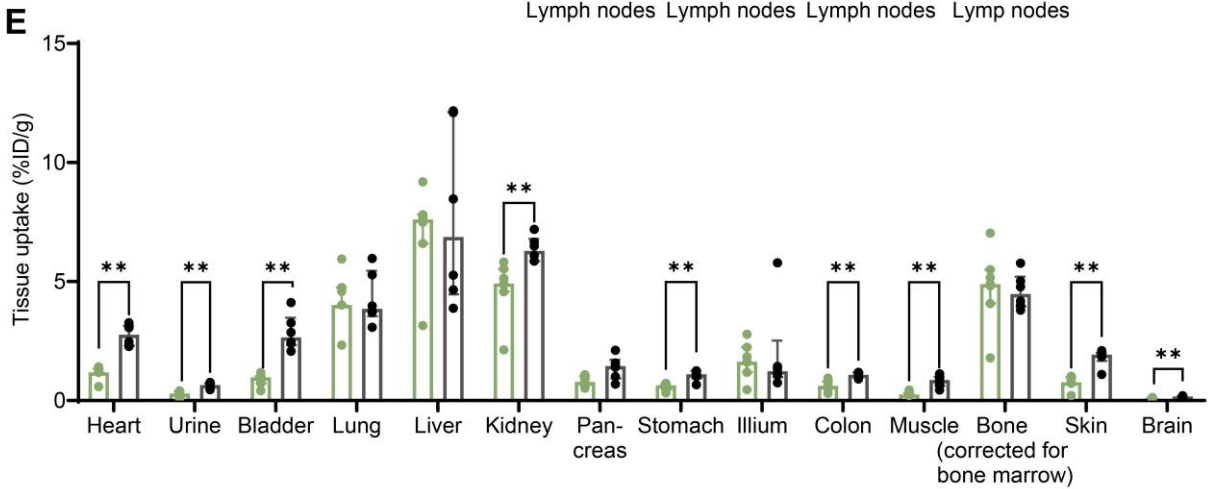
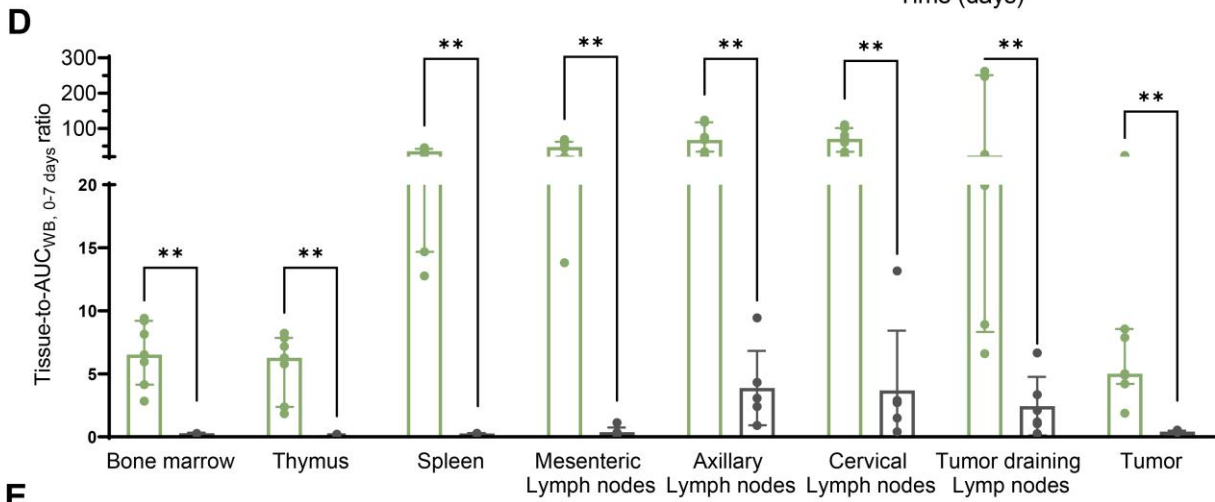
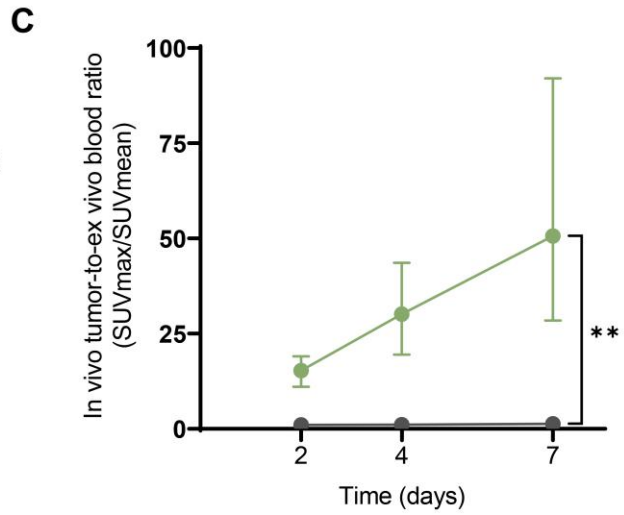
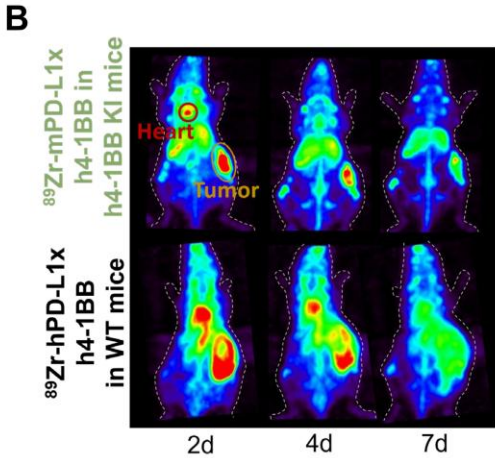
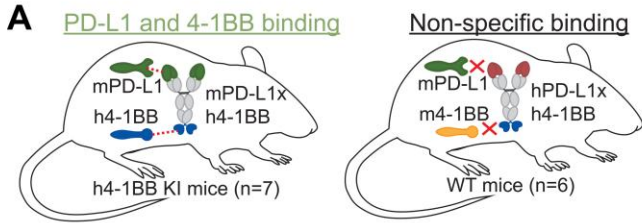


**Figure S5. Flow cytometry gating strategy used in internalization experiments.**

**(A)** Gating strategy for selecting healthy single tumor cells. **(B)** Gating strategy for selecting healthy single T-cells (CD3-positive cells) from PBMCs stimulated with ionomycin/PMA. **(C)** Gating strategy for selecting healthy single human T-cells from PBMCs stimulated with IL-2/PHA. Debris was removed in an FSC versus an SSC dot plot. Single cells were gated by plotting FSC-H versus area FSC-A, and live cells were gated based on a PI-A staining. CD3-positive human cells in the PBMC samples were gated on the anti-human Brilliant Violet™ 421 (BV421) CD3 staining and measured in the Pacific Blue-A. Binding/internalization of biotinylated hPD-Lxh4-1BB Mabcalin was detected

using Alexa Fluor™ 647 conjugated streptavidin within the live single tumor and T-cells and expressed as MFI. Samples were measured in triplicate and corrected for background fluorescence.

FSC: forward scatter; FSC-A: forward scatter area; FSC-H: forward scatter height; IL-2: interleukin 2; MFI: mean fluorescent intensity; Pacific Blue-A: pacific blue area; PBMCs: peripheral blood mononuclear cells; PHA: phytohemagglutinin; PI-A: propidium iodide area; PMA: phorbol myristate acetate; SSC: side scatter; SSC-A: side scatter area.

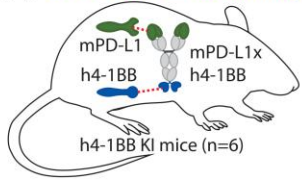


**Figure S6. Specificity of <sup>89</sup>Zr-mPD-L1xh4-1BB in MC38-tumor bearing h4-1BB KI mice dosed at 30 µg.**

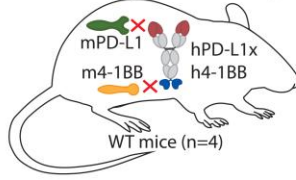
**(A)** Overview of experimental groups. Both Mabcalins are dosed at 30 µg. **(B)** Maximum intensity PET images of MC38 tumor-bearing h4-1BB KI or WT mice given 30 µg of <sup>89</sup>Zr-mPD-L1xh4-1BB or <sup>89</sup>Zr-hPD-L1xh4-1BB at 2, 4, and 7 days post-injection. A red circle highlights the heart, and a yellow circle the tumor. **(C)** In vivo tumor-to-ex vivo blood ratios at days 2, 4, and 7 post-injection. Ex vivo blood SUV was calculated based on blood samples after the PET scan. **(D)** Ex vivo uptake (tissue-to-AUC<sub>WB, 0-7 days</sub>) 7 days post-injection in lymphoid tissues and the tumor. **(E)** Ex vivo uptake (%ID/g) 7 days post-injection in organs in non-lymphoid tissues. Data are presented as median ± interquartile range.

%ID/g: percentage injected dose per gram tissue; AUC<sub>WB, 0-7 days</sub>: whole blood area under the curve from 0 to 7 days; d: day; h: human; h4-1BB KI mice: human 4-1BB knock-in mice; m: murine; SUV: standard uptake value; WT mice: wild-type mice; \*:  $P \leq 0.05$ ; \*\*:  $P \leq 0.01$ .

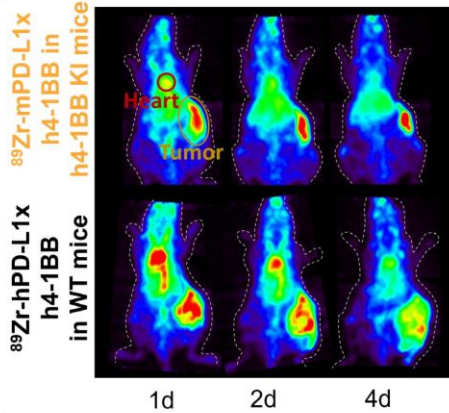
**A** PD-L1 and 4-1BB binding



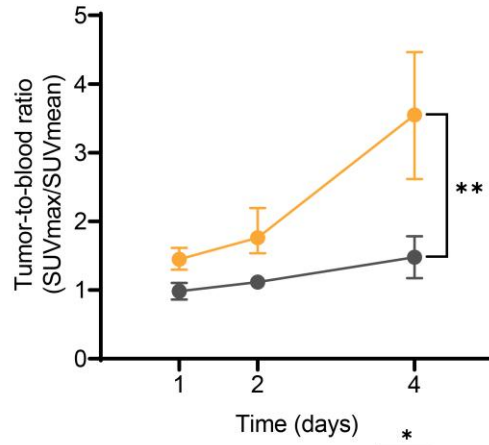
Non-specific binding



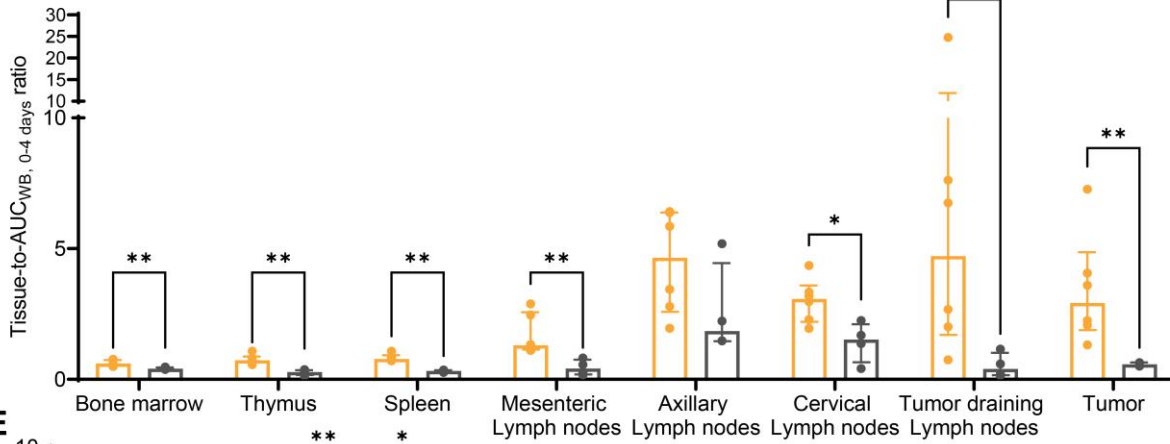
**B**



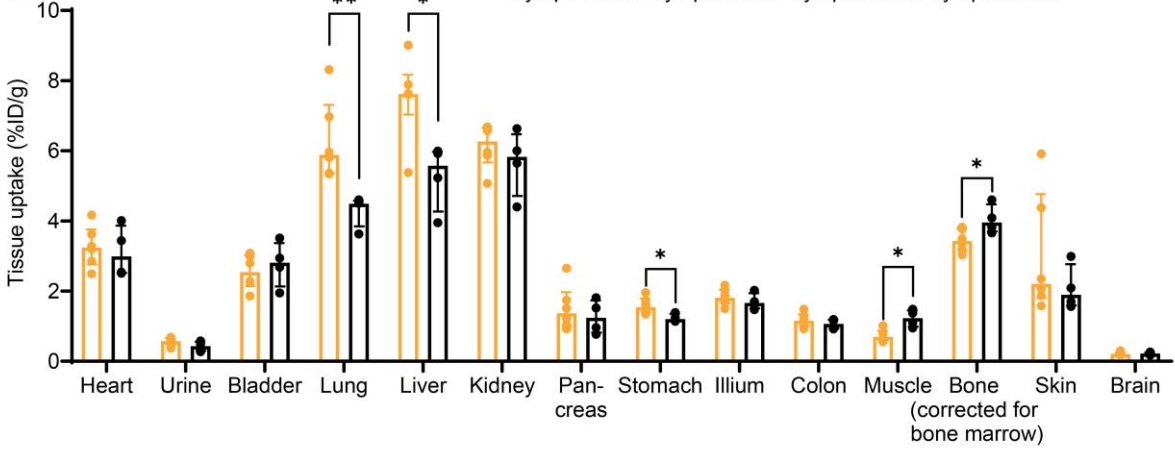
**C**



**D**



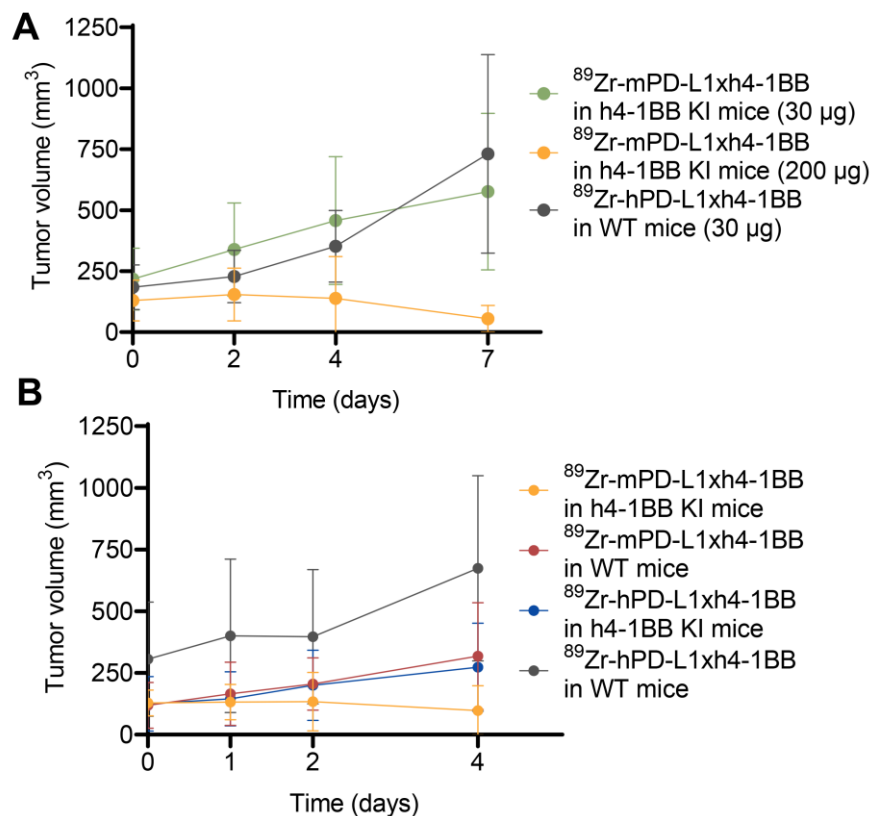
**E**



**Figure S7. Specificity of <sup>89</sup>Zr-mPD-L1xh4-1BB in MC38 tumor-bearing h4-1BB KI mice dosed at 200 µg.**

**(A)** Overview of experimental groups. Both Mabcalins are dosed at 200 µg. **(B)** Maximum intensity PET images of MC38 tumor-bearing h4-1BB KI or WT mice given 200 µg of <sup>89</sup>Zr-mPD-L1xh4-1BB or <sup>89</sup>Zr-hPD-L1xh4-1BB at 1, 2, and 4 days post-injection. A red circle highlights the heart, and a yellow circle the tumor. **(C)** In vivo uptake in tumor-to-blood ratios at days 1, 2, and 4 post-injection. **(D)** Ex vivo uptake (tissue-to-AUC<sub>WB, 0-4 days</sub>) 4 days post-injection in lymphoid tissues and the tumor. **(E)** Ex vivo uptake (%ID/g) 4 days post-injection in non-lymphoid tissues. Data are presented as median ± interquartile range.

%ID/g: percentage injected dose per gram tissue; AUC<sub>WB, 0-4 days</sub>: whole blood area under the curve from 0 to 4 days; d: day; h: human; h4-1BB KI: human 4-1BB knock-in mice; m: murine; SUV: standard uptake value; WT mice: wild-type mice; \*:  $P \leq 0.05$ ; \*\*:  $P \leq 0.01$ .



**Figure S8. Tumor size during the imaging time frame.**

**(A)** MC38 tumor volume in h4-1BB KI or WT mice that received <sup>89</sup>Zr-mPD-L1xh4-1BB or <sup>89</sup>Zr-hPD-L1xh4-1BB with a total protein dose of 30 or 200 µg at days 0, 2, 4, and 7 post-injection. **(B)** MC38 tumor volume in h4-1BB KI or WT mice given <sup>89</sup>Zr-mPD-L1xh4-1BB or <sup>89</sup>Zr-hPD-L1xh4-1BB with a total protein dose of 200 µg at days 0, 1, 2, and 4 post-injection. Data are presented as mean ± standard deviation.

H: human; h4-1BB KI mice: human 4-1BB knock-in mice; m: murine; WT mice: wild-type mice.

**Table S1. In vivo tumor uptake (SUVmax) and in vivo tumor-to-ex vivo blood ratio (SUVmax/SUVmean) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 2, 4 and 7 days post-injection.**

Data are presented as median ± interquartile range.

Time (days)		<sup>89</sup> Zr-mPD-L1xh4-1BB in h4-1BB KI mice (30 µg; n=7)	<sup>89</sup> Zr-mPD-L1xh4-1BB in h4-1BB KI mice (200 µg; n=5)	<sup>89</sup> Zr-hPD-L1xh4-1BB in WT mice (30 µg; n=6)
2	Tumor	2.58 (1.86 – 3.66)	2.83 (2.50 – 3.75)	NA
	In vivo tumor-to-ex vivo blood ratio	15.29 (11.06 – 19.02)	NA	1.08 (0.84 – 1.19)
4	Tumor	1.57 (1.09 – 3.06)	3.67 (2.97 – 5.33)	NA
	In vivo tumor-to-ex vivo blood ratio	30.09 (19.50 – 43.59)	NA	1.11 (1.01 – 1.15)
7	Tumor	1.20 (0.74 – 2.23)	4.99 (3.71 – 8.23)	NA
	In vivo tumor-to-ex vivo blood ratio	50.63 (28.45 – 92.04)	NA	1.31 (1.15 – 1.46)

NA, not applicable

**Table S2. In vivo tumor-to-blood ratio (SUVmax/SUVmean) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 1, 2 and 4 days post-injection.**

Data are presented as median ± interquartile range.

Time (days)	<sup>89</sup> Zr-mPD-L1xh4-1BB in h4-1BB KI mice (200 µg; n=6)	<sup>89</sup> Zr-mPD-L1xh4-1BB in WT mice (200 µg; n=4)	<sup>89</sup> Zr-hPD-L1xh4-1BB in 4-BB KI mice (200 µg; n=5)	<sup>89</sup> Zr-hPD-L1xh4-1BB in WT mice (200 µg; n=4)
1	1.45 (1.30 – 1.61)	0.94 (0.78 – 1.16)	0.98 (0.83 – 1.20)	0.97 (0.88 – 1.11)
2	1.76 (1.54 – 2.19)	1.15 (0.94 – 1.28)	1.25 (1.06 – 1.40)	1.09 (1.03 – 1.23)
4	3.55 (2.62 – 4.47)	1.64 (1.40 – 2.01)	1.41 (1.35 – 1.55)	1.48 (1.18 – 1.78)

**Table S3. Absolute ex vivo uptake (%ID/g) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 7 days post-injection in lymphoid tissues and the tumor.**

Data are presented as median ± interquartile range.

Tissue	<sup>89</sup> Zr-mPD-L1x4-1BB in h4-1BB KI mice (30 µg; n=7)	<sup>89</sup> Zr-mPD-L1x4-1BB in h4-1BB KI mice (200 µg; n=5)	<sup>89</sup> Zr-hPD-L1x4-1BB in WT mice (30 µg; n=6)
Bone marrow	2.72 (1.82 – 3.09)	3.48 (2.81 – 5.07)	4.10 (3.63 – 5.31)
Thymus	2.40 (2.12 – 2.67)	4.22 (3.85 – 5.72)	2.38 (1.44 – 2.97)
Spleen	12.59 (10.40 – 15.62)	8.98 (6.98 – 10.56)	4.04 (3.48 – 5.05)
Mesenteric lymph nodes	17.58 (14.59 – 24.45)	10.11 (7.74 – 12.67)	3.72 (2.89 – 8.07)
Axillary lymph nodes	28.50 (20.13 – 40.26)	32.15 (12.17 – 45.36)	54.14 (27.77 – 82.23)
Cervical lymph nodes	32.97 (18.47 – 38.32)	20.75 (14.49 – 73.37)	31.50 (18.70 – 96.29)
Tumor draining lymph nodes	6.82 (2.67 – 83.30)	16.48 (7.36 – 36.33)	26.02 (11.41 – 66.81)
Tumor	2.53 (1.53 – 5.12)	19.03 (10.27 – 54.09)	6.11 (5.35 – 7.73)

**Table S4. Ex vivo uptake (tissue-to-blood AUC<sub>WB, 0-7 days</sub>) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 7 days post-injection in lymphoid tissues and the tumor.**

Data are presented as median ± interquartile range.

Tissue	<sup>89</sup> Zr-mPD-L1x4-1BB in h4-1BB KI mice (30 µg; n=7)	<sup>89</sup> Zr-mPD-L1x4-1BB in h4-1BB KI mice (200 µg; n=5)	<sup>89</sup> Zr-hPD-L1x4-1BB in WT mice (30 µg; n=6)
Bone marrow	6.52 (4.14 – 9.20)	0.63 (0.62 – 0.70)	0.28 (0.23 – 0.33)
Thymus	6.28 (2.38 – 7.86)	0.87 (0.71 – 0.89)	0.14 (0.10 – 0.21)
Spleen	35.66 (14.68 – 42.59)	1.48 (1.07 – 2.18)	0.28 (0.23 – 0.30)
Mesenteric lymph nodes	47.77 (20.05 – 62.26)	1.71 (1.24 – 2.68)	0.25 (0.16 – 0.54)
Axillary lymph nodes	67.63 (34.64 – 117.31)	3.47 (2.58 – 8.31)	3.07 (2.04 – 5.60)
Cervical lymph nodes	70.90 (33.79 – 101.06)	4.10 (3.03 – 8.77)	2.11 (1.19 – 5.46)
Tumor draining lymph nodes	23.25 (8.33 – 251.01)	4.06 (1.22 – 6.21)	1.64 (0.84 – 4.18)
Tumor	5.01 (4.20 – 8.56)	2.93 (2.22 – 9.24)	0.42 (0.37 – 0.46)

**Table S5. Absolute ex vivo uptake (%ID/g) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 4 days post-injection in lymphoid tissues and the tumor.**

Data are presented as median ± interquartile range.

Tissue	<sup>89</sup> Zr-mPD-L1x4-1BB in h4-1BB KI mice (200 µg; n=6)	<sup>89</sup> Zr-mPD-L1x4-1BB in WT mice (200 µg; n=4)	<sup>89</sup> Zr-hPD-L1x4-1BB in 4-BB KI mice (200 µg; n=5)	<sup>89</sup> Zr-hPD-L1x4-1BB in WT mice (200 µg; n=4)
Bone marrow	4.44 (3.74 – 5.30)	4.50 (4.18 – 5.56)	5.07 (4.52 – 5.58)	4.86 (4.14 – 5.62)
Thymus	5.13 (4.75 – 5.66)	4.58 (4.24 – 5.09)	3.19 (2.96 – 4.61)	3.56 (1.93 – 3.79)
Spleen	5.51 (4.98 – 6.01)	5.22 (4.76 – 6.03)	3.27 (3.14 – 4.35)	3.84 (3.01 – 4.30)
Mesenteric lymph nodes	9.69 (8.18 – 13.97)	4.48 (2.49 – 8.05)	4.30 (3.66 – 13.92)	4.36 (2.33 – 9.08)
Axillary lymph nodes	27.69 (17.87 – 44.21)	19.41 (12.48 – 25.11)	51.31 (32.24 – 101.3)	21.00 (16.80 – 54.24)
Cervical lymph nodes	20.40 (14.42 – 25.75)	14.45 (12.60 – 33.75)	26.44 (18.70 – 42.19)	16.33 (7.67 – 25.98)
Tumor draining lymph nodes	32.80 (8.49 – 87.30)	6.05 (3.52 – 9.82)	12.00 (6.91 – 15.86)	4.80 (1.67 – 12.96)
Tumor	21.13 (10.19 – 34.56)	7.39 (6.15 – 8.43)	9.72 (7.20 – 11.32)	6.84 (5.39 – 7.79)

**Table S6. Ex vivo uptake (tissue-to-blood AUC<sub>WB, 0-4 days</sub>) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 4 days post-injection in lymphoid tissues and the tumor.**

Data are presented as median ± interquartile range.

Tissue	<sup>89</sup> Zr-mPD-L1x4-1BB in h4-1BB KI mice (200 µg; n=6)	<sup>89</sup> Zr-mPD-L1x4-1BB in WT mice (200 µg; n=4)	<sup>89</sup> Zr-hPD-L1x4-1BB in 4-BB KI mice (200 µg; n=5)	<sup>89</sup> Zr-hPD-L1x4-1BB in WT mice (200 µg; n=4)
Bone marrow	0.61 (0.57 – 0.74)	0.55 (0.51 – 0.60)	0.42 (0.41 – 0.45)	0.41 (0.38 – 0.46)
Thymus	0.73 (0.66 – 0.87)	0.54 (0.48 – 0.60)	0.30 (0.26 – 0.36)	0.28 (0.17 – 0.35)
Spleen	0.78 (0.70 – 0.92)	0.59 (0.54 – 0.75)	0.29 (0.28 – 0.34)	0.32 (0.28 – 0.35)
Mesenteric lymph nodes	1.30 (1.14 – 2.57)	0.48 (0.30 – 1.00)	0.33 (0.32 – 1.25)	0.41 (0.19 – 0.75)
Axillary lymph nodes	4.65 (2.58 – 6.38)	2.21 (1.59 – 2.77)	4.70 (2.84 – 8.01)	1.85 (1.46 – 4.45)
Cervical lymph nodes	3.07 (2.20 – 3.59)	1.61 (1.46 – 4.22)	2.45 (1.56 – 3.55)	1.52 (0.65 – 2.11)
Tumor draining lymph nodes	4.71 (1.70 – 11.91)	0.64 (0.42 – 1.22)	1.05 (0.62 – 1.26)	0.40 (0.16 – 1.02)
Tumor	2.92 (1.88 – 4.86)	0.84 (0.78 – 0.91)	0.72 (0.64 – 0.99)	0.57 (0.49 – 0.65)

**Table S7. Ex vivo uptake (tissue-to-blood AUC<sub>WB, 0-7 days</sub>) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 7 days post-injection in non-lymphoid tissues.**

Data are presented as median ± interquartile range.

<b>Tissue</b>	<b><sup>89</sup>Zr-mPD-L1x4-1BB in h4-1BB KI mice (30 µg; n=7)</b>	<b><sup>89</sup>Zr-mPD-L1x4-1BB in h4-1BB KI mice (200 µg; n=5)</b>	<b><sup>89</sup>Zr-hPD-L1x4-1BB in WT mice (30 µg; n=6)</b>
<b>Heart</b>	1.19 (1.18 – 1.34)	2.24 (1.56 – 2.67)	2.77 (2.31 – 3.14)
<b>Urine</b>	0.30 (0.17 – 0.38)	0.60 (0.48 – 1.10)	0.65 (0.55 – 0.72)
<b>Bladder</b>	0.98 (0.72 – 1.02)	1.61 (0.92 – 1.91)	2.66 (2.33 – 3.48)
<b>Lung</b>	4.01 (4.01 – 4.75)	5.75 (3.88 – 6.38)	3.86 (3.55 – 5.45)
<b>Liver</b>	7.61 (6.60 – 7.81)	9.76 (8.69 – 10.96)	6.87 (4.46 – 12.12)
<b>Kidney</b>	4.92 (4.57 – 5.53)	5.83 (4.73 – 6.11)	6.29 (5.86 – 6.79)
<b>Pancreas</b>	0.79 (0.64 – 1.03)	0.72 (0.62 – 0.88)	1.45 (0.93 – 1.71)
<b>Stomach</b>	0.64 (0.45 – 0.68)	0.98 (0.63 – 1.28)	1.11 (0.93 – 1.25)
<b>Illium</b>	1.63 (1.19 – 2.24)	1.78 (1.07 – 2.07)	1.24 (0.99 – 2.52)
<b>Colon</b>	0.62 (0.51 – 0.80)	0.81 (0.72 – 1.03)	1.09 (1.00 – 1.18)
<b>Muscle</b>	0.26 (0.21 – 0.32)	0.60 (0.32 – 0.74)	0.87 (0.61 – 1.00)
<b>Bone (corrected for bone marrow)</b>	4.89 (4.08 – 5.50)	5.21 (4.08 – 6.05)	4.48 (3.95 – 5.21)
<b>Skin</b>	0.77 (0.72 – 0.97)	0.92 (0.75 – 1.56)	1.93 (1.66 – 2.00)
<b>Brain</b>	0.08 (0.07 – 0.12)	0.15 (0.12 – 0.19)	0.17 (0.15 – 0.19)

**Table S8. Ex vivo uptake (tissue-to-blood AUC<sub>WB, 0-7 days</sub>) of <sup>89</sup>Zr-mPD-L1x4-1BB and <sup>89</sup>Zr-hPD-L1x4-1BB in h4-1BB KI or WT mice at 4 days post-injection in non-lymphoid tissues.**

Data are presented as median ± interquartile range.

<b>Tissue</b>	<b><sup>89</sup>Zr-mPD-L1xh4-1BB in h4-1BB KI mice (200 µg; n=6)</b>	<b><sup>89</sup>Zr-mPD-L1xh4-1BB in WT mice (200 µg; n=4)</b>	<b><sup>89</sup>Zr-hPD-L1xh4-1BB in 4-BB KI mice (200 µg; n=5)</b>	<b><sup>89</sup>Zr-hPD-L1xh4-1BB in WT mice (200 µg; n=4)</b>
<b>Heart</b>	3.24 (2.76 – 3.76)	2.68 (2.40 – 2.75)	3.01 (2.68 – 4.53)	2.99 (2.52 – 3.87)
<b>Urine</b>	0.58 (0.48 – 0.67)	0.49 (0.37 – 0.52)	0.32 (0.29 – 0.70)	0.43 (0.30 – 0.56)
<b>Bladder</b>	2.54 (2.15 – 3.02)	2.95 (2.80 – 3.04)	2.90 (2.72 – 4.98)	2.81 (2.13 – 3.37)
<b>Lung</b>	5.89 (5.36 – 7.31)	4.63 (4.23 – 5.50)	5.17 (4.33 – 5.68)	4.49 (3.84 – 4.58)
<b>Liver</b>	7.62 (7.03 – 8.17)	8.31 (6.51- 11.10)	6.90 (5.84 – 7.25)	5.58 (4.27 – 5.97)
<b>Kidney</b>	6.27 (5.67 – 6.66)	5.66 (5.29 – 6.22)	6.33 (4.73 – 7.01)	5.83 (4.71 – 6.47)
<b>Pancreas</b>	1.37 (1.01 – 1.97)	0.93 (0.82 – 0.93)	1.77 (0.99 – 2.13)	1.24 (0.82 – 1.74)
<b>Stomach</b>	1.54 (1.39 – 1.79)	1.42 (1.36 – 1.83)	1.62 (1.14 – 1.79)	1.21 (1.15 – 1.36)
<b>Illium</b>	1.81 (1.64 – 2.05)	1.91 (1.58 – 2.33)	1.63 (1.39 – 1.87)	1.66 (1.51 – 1.94)
<b>Colon</b>	1.16 (1.00 – 1.34)	1.11 (1.06 – 1.53)	1.23 (0.96 – 1.78)	1.07 (0.93 – 1.18)
<b>Muscle</b>	0.70 (0.60 – 0.87)	0.78 (0.49 – 1.47)	0.90 (0.77 – 1.37)	1.23 (0.99 – 1.45)
<b>Bone (corrected for bone marrow)</b>	3.43 (3.15 – 3.78)	3.23 (2.87 – 3.60)	3.56 (2.66 – 4.00)	3.96 (3.70 – 4.47)
<b>Skin</b>	2.21 (1.80 – 4.76)	2.47 (2.32 – 2.76)	2.59 (2.26 – 4.86)	1.90 (1.60 – 2.77)
<b>Brain</b>	0.21 (0.18 – 0.24)	0.20 (0.17 – 0.23)	0.20 (0.18 – 0.24)	0.22 (0.18 – 0.25)