## HLE BiTE molecule conjugation, labeling and characterization

A cell-based assay served to evaluate binding of the MSLN HLE BiTE molecule to its targets. Concentrations from $10^{-3}$ nM to $10^{3}$ nM of the MSLN HLE BiTE were incubated for 1 hour at 4 ˚C with 2.5 x $10^{5}$ murine T-cells or 4T1 cells. Murine T-cells were obtained by negative selection with the Pan T-Cell Isolation Kit II, mouse (Miltenyi Biotec). After incubation for 1 hour at 4 ˚C with the MSLN HLE BiTE, cells were washed and incubated with a secondary antibody, either goat anti-mouse IgG-APC (Jackson ImmunoReseach) or goat anti-mouse IgG - AF647 (Invitrogen). Cells were gated for live cells with fixable viability dye eFluor 506 (Affymetrix, eBioscience). Data was acquired by BD LSRFortessa Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo v10). Mesothelin expression was quantified on 4T1 and OVCAR8 cells with a standard receptor quantitation QIFIKIT® assay (Dako).

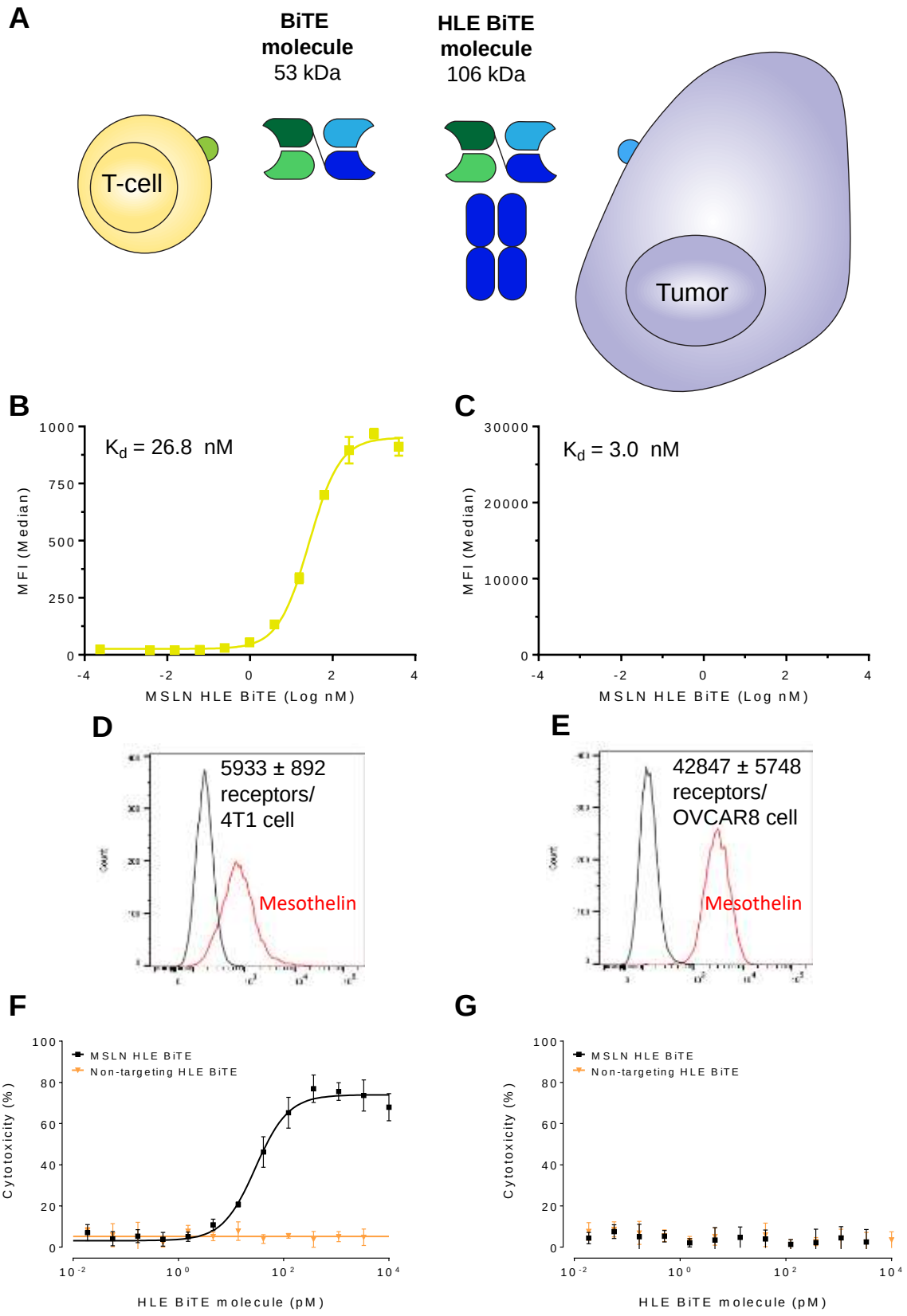
Specific cytotoxicity of MSLN HLE BiTE and the control HLE BiTE molecule was studied adding concentrations of $10^{-3}$ pM to $10^{4}$ pM of either molecule to mouse T-cells and $10^{4}$ 4T1 or B16F10 tumor cells in a ratio of 10:1. The melanoma cell line B16F10 (ATCC) was cultured in DMEM medium (Biochrom) containing 10% fetal calf serum (Invitrogen). Read-outs were propidium iodide-positive tumor cells for cytotoxicity and CD69-positive T-cells for T-cell activation. Data was acquired by FACS Canto II (BD Biosciences) and sigmoidal curves were generated using GraphPad Prism 7.

Both HLE BiTE molecules were conjugated as previously described (*13*). In short, tetrafluorophenol-*N*-succinyl-desferrioxamine-Fe (TFP-*N*-suc-DFO-Fe; ABX) was conjugated to the MSLN HLE BiTE and the control HLE BiTE. Conjugation efficiency and protein purity were evaluated by size exclusion ultra-performance liquid chromatography (SE-UPLC, Waters) with a dual-wavelength absorbance detector (280 nm versus 430

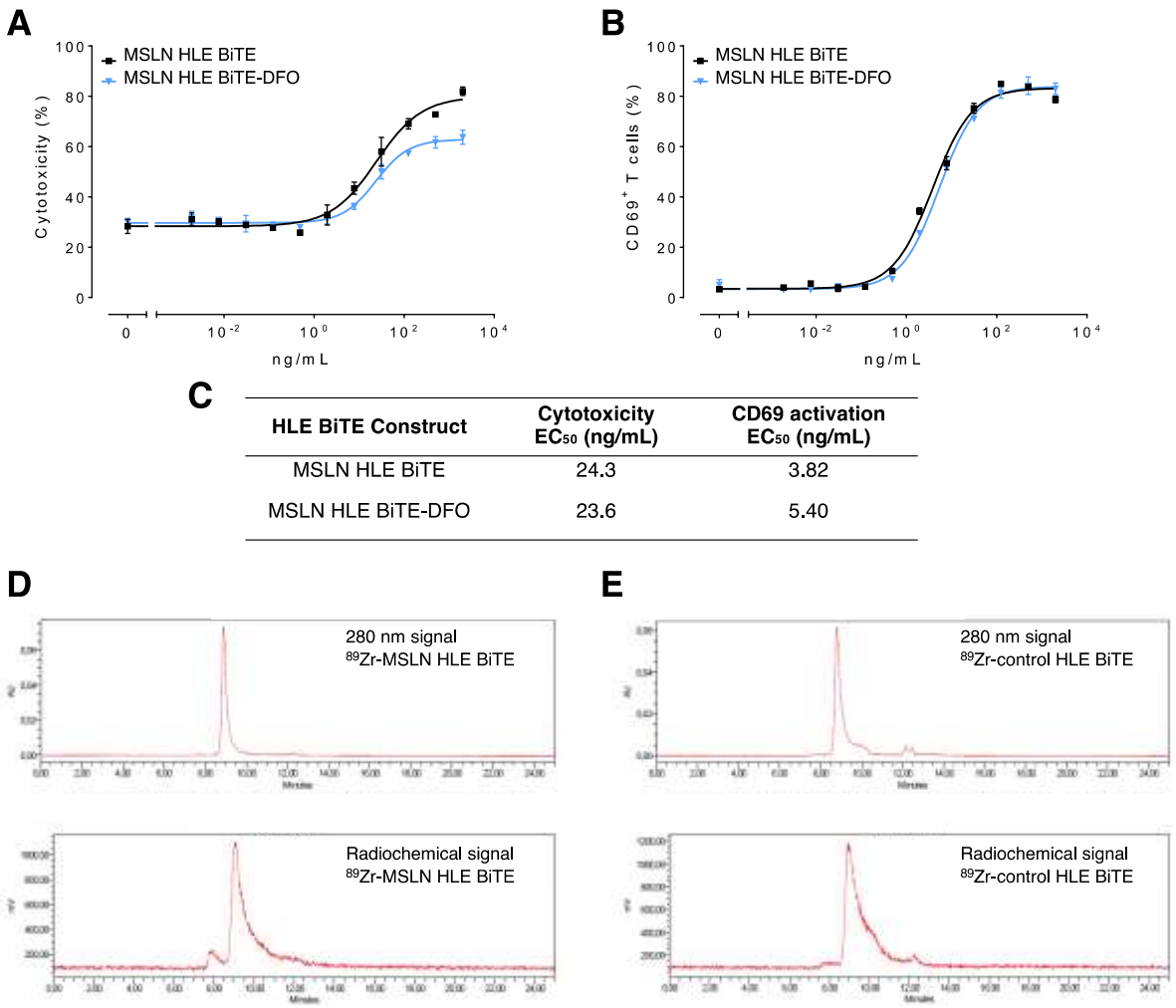
nm). A TSKgel G3000SW$_{XL}$ column (Tosoh) and phosphate-buffered saline (140 mmol/L NaCl, 9 mmol/L $Na_2HPO_4$, 1.3 mmol/L $NaH_2PO_4$; pH 7.4) as mobile phase were used. The conjugate with a concentration of 1 mg/mL was stored at -80 ˚C. Stability by assessing the formation of low and high molecular weight species was determined by SE-UPLC analysis. Maintained immunoreactivity for both arms was studied by functional cell-based assays: cytotoxicity and T-cell activation. Concentrations of $10^{-3}$ ng/mL to $10^3$ ng/mL conjugated or unmodified HLE BiTE molecules were added to murine T-cells and $10^4$ 4T1 tumor cells in a ratio of 10:1. Read-outs were propidium iodide-positive tumor cells for cytotoxicity and CD69-positive T-cells for T-cell activation. Data was acquired by FACS Canto II.

The conjugated HLE BiTE molecules were labeled with $^{89}$Zr, as described previously (*25*). Radiochemical purity was evaluated by a trichloroacetic acid precipitation assay and SE-UPLC analysis. No visible particles were detected. Conjugated MSLN HLE BiTE and conjugated control HLE BiTE were labeled with $^{89}$Zr with >95% radiochemical purity at a specific activity of 400 - 500 MBq/mg.

A radiolabeled protein dose of 10 µg $^{89}$Zr-MSLN HLE BiTE or 10 µg $^{89}$Zr-control HLE BiTE was prepared for each dose group. A cold protein dose of the respective unlabeled parental HLE BiTE was added to the hot dose to reach a final protein dose of 50 µg or 200 µg.

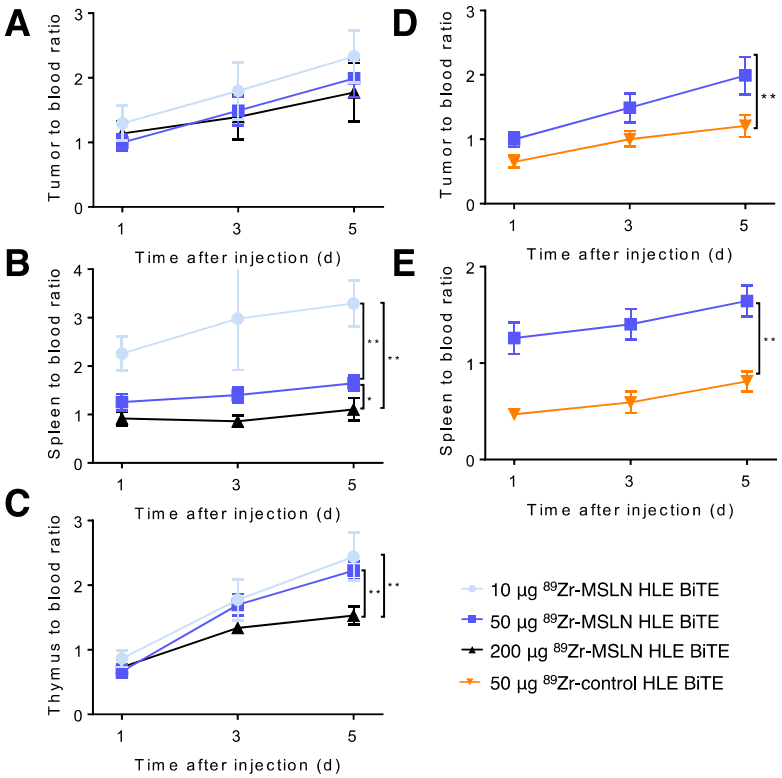

A
BiTE molecule
53 kDa
HLE BiTE molecule
106 kDa
T-cell
Tumor
B
$K_d$ = 26.8 nM
MFI (Median)
MSLN HLE BiTE (Log nM)
C
$K_d$ = 3.0 nM
MFI (Median)
MSLN HLE BiTE (Log nM)
D
5933 ± 892 receptors/ 4T1 cell
Mesothelin
E
42847 ± 5748 receptors/ OVCAR8 cell
Mesothelin
F
MSLN HLE BiTE
Non-targeting HLE BiTE
Cytotoxicity (%)
HLE BiTE molecule (pM)
G
MSLN HLE BiTE
Non-targeting HLE BiTE
Cytotoxicity (%)
HLE BiTE molecule (pM)

**Supplemental Figure 1.** A, Simplified schematic overview of a canonical BiTE and HLE BiTE molecule. Both bind to T-cell and tumor, but the HLE BiTE has an Fc-domain increasing its size. B, Binding assay of the MSLN HLE BiTE with CD3 on murine T-cells. C, Binding assay of the MSLN HLE BiTE with mesothelin on 4T1 cells. Data is presented as mean ± standard deviation. D and E, Mesothelin expression (red) on 4T1 cells (D) and on OVCAR8 cells (E) expressed as mean ± standard deviation (n = 2). F and G, cytotoxicity assay with T-cells: 4T1-tumor cells (F) and B16F10 (G, mesothelin negative) incubated at a ratio of 10:1.

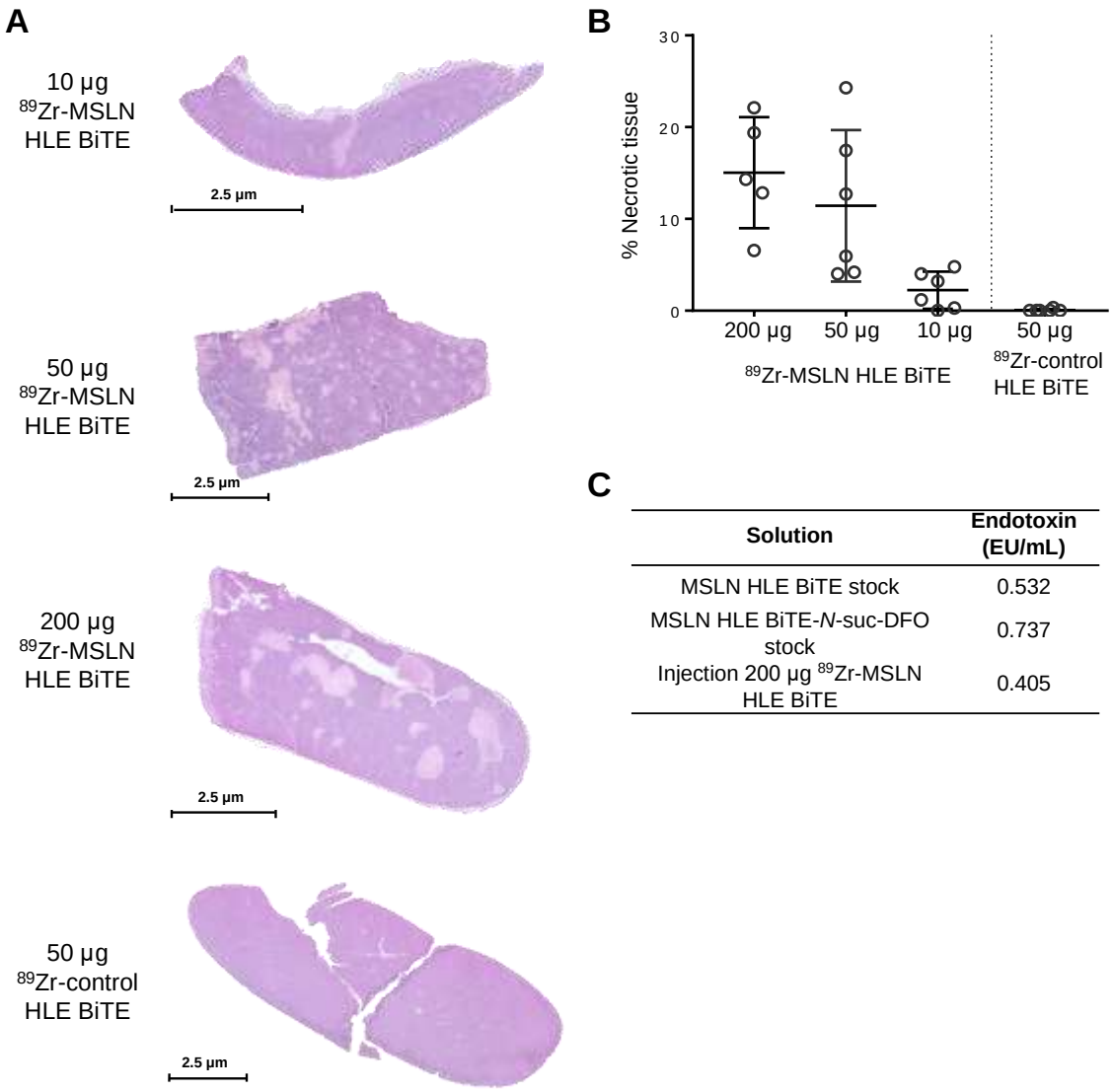



| HLE BiTE Construct | Cytotoxicity $EC_{50}$ (ng/mL) | CD69 activation $EC_{50}$ (ng/mL) |
|---|---|---|
| MSLN HLE BiTE | 24.3 | 3.82 |
| MSLN HLE BiTE-DFO | 23.6 | 5.40 |

**Supplemental Figure 2.** Characterization of $^{89}$Zr-HLE BiTE. Functionality assessed by cell-based assays with T-cells: 4T1-tumor cells incubated at a ratio of 10:1. A, Cytotoxicity assay. B, T-cell activation by CD69 expression. Data is presented as mean ± standard deviation. C, Quantification of sigmoidal curves of A and B. D, UPLC protein signal (280 nm) and radiochemical signal of $^{89}$Zr-MSLN HLE BiTE. E, UPLC protein signal (280 nm) and radiochemical signal of $^{89}$Zr-control HLE BiTE.



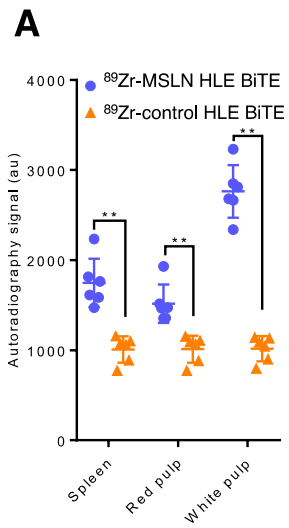

**Supplemental Figure 3.** Organ-to-blood ratios of 10, 50 or 200 µg $^{89}$Zr-MSLN HLE A, Tumor. B, Spleen. C, Thymus. Organ-to-blood ratios of 50 µg $^{89}$Zr-MSLN HLE BiTE compared with 50 µg $^{89}$Zr-control HLE BiTE D, Tumor. E, Spleen.



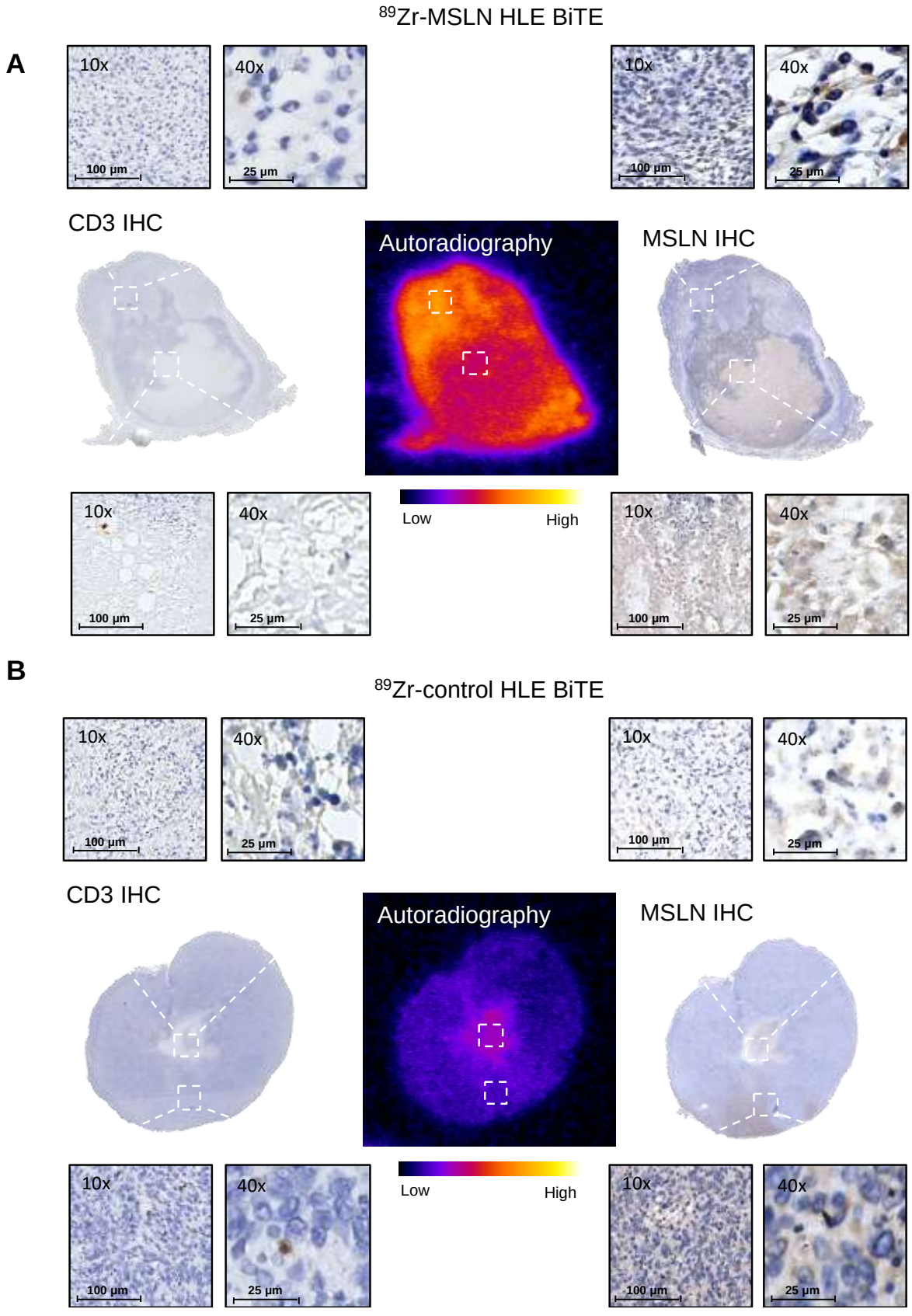

C

| Solution | Endotoxin (EU/mL) |
|---|---|
| MSLN HLE BiTE stock | 0.532 |
| MSLN HLE BiTE-*N*-suc-DFO stock | 0.737 |
| Injection 200 µg $^{89}$Zr-MSLN HLE BiTE | 0.405 |

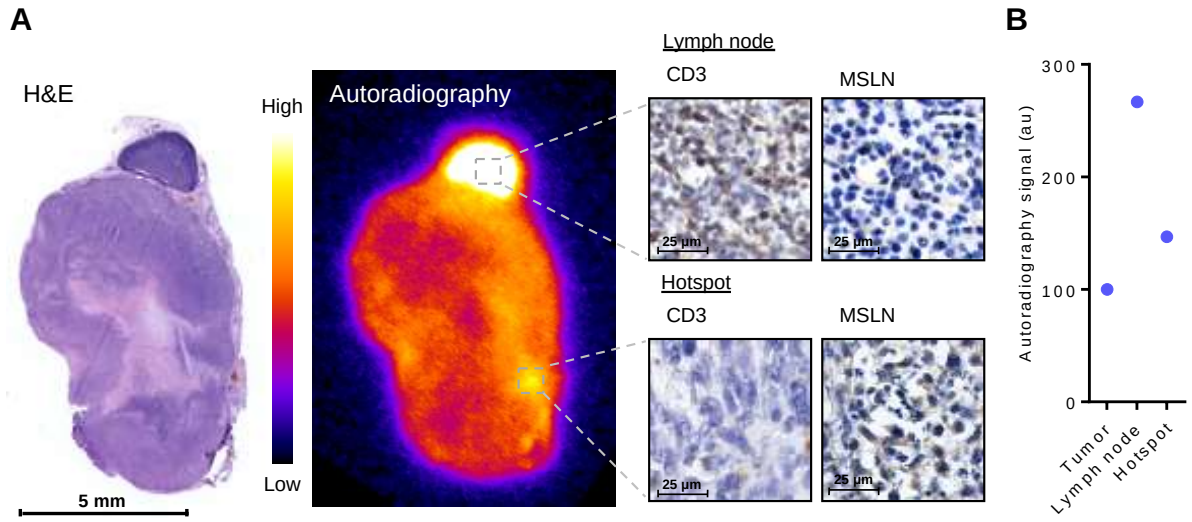
**Supplemental Figure 4.** Analysis of the liver of 4T1-tumor bearing mice. A, Representative H&E stainings from the different tracer dose groups. B, Necrotic area of liver tissue expressed as a percentage of the area of the entire H&E stained slice. Data is presented as mean ± standard deviation. C, Endotoxin values measured by a limulus amebocyte lysate assay.




**Supplemental Figure 5.** Red and white pulp quantified from autoradiography data from Figure 5A, normalized for injected activity. Data expressed as arbitrary units (au) presented as mean ± standard deviation; *: $P \leq 0.05$, **: $P \leq 0.01$.


$^{89}$Zr-MSLN HLE BiTE
A
10x
40x
100 µm
25 µm
CD3 IHC
Autoradiography
MSLN IHC
Low
High
B
$^{89}$Zr-control HLE BiTE

**Supplemental Figure 6.** Magnifications from Figure 5A and Figure 5B, *Ex vivo* analysis of spleen and tumor tissue (4T1) 5 days after injection of 50 µg $^{89}$Zr-MSLN HLE BiTE or 50 µg $^{89}$Zr-control HLE BiTE. Left side, CD3 IHC. Right side, Mesothelin IHC.




**Supplemental Figure 7.** *Ex vivo* microscopic analysis of tumor tissue with adjacent lymph node 5 days after injection of 50 µg $^{89}$Zr-MSLN HLE BiTE. A, From left to right, hematoxylin and eosin (H&E) staining and corresponding autoradiography. Next, immunohistochemistry (IHC) on CD3 and mesothelin (MSLN) expression. B, Quantified autoradiography data showing higher signal in regions of interest in lymph node than in the tumor and tumor hotspot. Data expressed as arbitrary units (au).

**Supplemental Table 1.** *Ex vivo* biodistribution data of the three dose groups 5 days after $^{89}$Zr-MSNL HLE BiTE tracer injection in 4T1-tumor bearing syngeneic mice.

| Tissue | 10 µg $^{89}$Zr-MSLN HLE BiTE | 50 µg $^{89}$Zr-MSLN HLE BiTE | 200 µg $^{89}$Zr-MSLN HLE BiTE | ANOVA/ Kruskal-Wallis *P* |
|---|---|---|---|---|
| **Blood** | 2.72 (2.49 to 4.12) | 4.56 (4.17 to 5.83) | 5.51 (4.66 to 6.06) | 0.013 |
| **Plasma** | 4.68 (4.68 to 5,18) | 7.96 (7.56 to 8.21) | 10.13 (8.55 to 12.05) | < 0.001 |
| **Heart** | 2.25 (2.07 to 2.50) | 3.73 (3.34 to 3.96) | 2.59 (2.40 to 2.95) | < 0.001 |
| **Lung** | 7.47 (4.90 to 9.31) | 10.11 (8.78 to 12.28) | 7.35 (5.43 to 8.31) | 0.030 |
| **Liver** | 9.16 (8.06 to 9.45) | 7.77 (6.92 to 8.28) | 5.92 (4.62 to 7.86) | 0.005 |
| **Kidney** | 6.94 (6.52 to 7.11) | 7.80 (6.94 to 8.21) | 7.09 (6.28 to 7.69) | ns |
| **Pancreas** | 2.58 (1.81 to 2.93) | 2.88 (2.30 to 3.65) | 2.35 (1.92 to 2.59) | ns |
| **Spleen** | 10.12 (9.53 to 12.71) | 8.83 (5.95 to 12.17) | 4.46 (3.85 to 6.75) | 0.003 |
| **Thymus** | 28.95 (19.88 to 34.20) | 40.78 (36.15 to 65.11) | 28.49 (17.97 to 49.90) | ns |
| **Mesenteric LNs** | 31.97 (21.16 to 39.89) | 22.57 (20.58 to 24.67) | 12.03 (10.30 to 12.67) | < 0.001 |
| **Stomach** | 1.39 (0.91 to 1.65) | 1.55 (1.44 to 1.64) | 1.65 (1.25 to 1.84) | ns |
| **Duodenum** | 1.42 (0.82 to 1.99) | 2.02 (1.59 to 2.42) | 1.55 (1.29 to 2.03) | ns |
| **Ileum** | 2.11 (2.06 to 2.49) | 2.42 (2.09 to 2.83) | 1.64 (1.32 to 2.52) | ns |
| **Colon** | 1.38 (1.18 to 1.49) | 1.88 (1.53 to 2.50) | 1.56 (1.23 to 1.68) | ns |
| **Adipose tissue** | 4.36 (2.87 to 5.56) | 4.46 (3.37 to 5.98) | 3.10 (2.05 to 4.35) | ns |
| **Muscle** | 0.40 (0.30 to 0.65) | 0.66 (0.60 to 1.13) | 0.67 (0.58 to 1.41) | 0.032 |
| **Brain** | 0.15 (0.13 to 0.17) | 0.19 (0.17 to 0.23) | 0.26 (0.22 to 0.39) | < 0.001 |
| **Skin** | 1.40 (0.67 to 2.04) | 1.81 (1.40 to 2.23) | 1.65 (1.40 to 2.78) | ns |
| **Bone** | 5.00 (4.22 to 6.62) | 4.09 (3.39 to 4.70) | 3.75 (3.33 to 4.67) | ns |
| **Bone marrow** | 5.25 (4.25 to 6.05) | 6.44 (6.22 to 7.96) | 8.16 (7.79 to 9.43) | ns |
| **Tumor** | 8.76 (8.31 to 12.31) | 10.59 (9.66 to 11.95) | 9.52 (9.28 to 9.96) | ns |

Data is presented as median % ID/g values with interquartile range from 10 µg $^{89}$Zr-MSLN HLE BiTE (n = 6), 50 µg $^{89}$Zr-MSLN HLE BiTE (n = 6) and 200 µg $^{89}$Zr-MSLN HLE BiTE (n = 5). An analysis of variance was performed with the Kruskal-Wallis test, *P*-values < 0.05 are shown. LN = lymph node, ns= non-significant.

**Supplemental Table 2.** *Ex vivo* biodistribution data of 50 µg $^{89}$Zr-MSLN HLE BiTE (n = 6) and 50 µg $^{89}$Zr-control HLE BiTE (n = 6) 5 days after tracer injection in 4T1-tumor bearing syngeneic mice.

| Tissue | 50 µg $^{89}$Zr-MSLN HLE BiTE | 50 µg $^{89}$Zr-control HLE BiTE | Mann-Whitney *P* |
|---|---|---|---|
| **Blood** | 4.56 (4.17 to 5.83) | 5.33 (4.78 to 6.06) | ns |
| **Plasma** | 7.96 (7.56 to 8.21) | 10.30 (7.89 to 11.80) | ns |
| **Heart** | 3.73 (3.34 to 3.96) | 1.81 (1.58 to 2.51) | 0.002 |
| **Lung** | 10.11 (8.78 to 12.28) | 3.24 (2.74 to 4.48) | 0.002 |
| **Liver** | 7.77 (6.92 to 8.28) | 4.40 (3.94 to 4.65) | 0.002 |
| **Kidney** | 7.80 (6.94 to 8.21) | 4.62 (4.27 to 6.10) | 0.004 |
| **Pancreas** | 2.88 (2.30 to 3.65) | 0.71 (0.41 to 1.19) | 0.002 |
| **Spleen** | 8.83 (5.95 to 12.17) | 2.47 (2.15 to 2.62) | 0.002 |
| **Thymus** | 40.78 (36.15 to 65.11) | 1.68 (1.30 to 2.15) | 0.002 |
| **Mesenteric LNs** | 22.57 (20.58 to 24.67) | 1.66 (1.29 to 1.82) | 0.002 |
| **Stomach** | 1.55 (1.44 to 1.64) | 1.05 (0.97 to 1.21) | 0.002 |
| **Duodenum** | 2.02 (1.59 to 2.42) | 0.83 (0.64 to 0.98) | 0.002 |
| **Ileum** | 2.42 (2.09 to 2.83) | 0.86 (0.70 to 0.94) | 0.002 |
| **Colon** | 1.88 (1.53 to 2.50) | 0.76 (0.48 to 0.79) | 0.002 |
| **Adipose tissue** | 4.46 (3.37 to 5.98) | 0.86 (0.65 to 3.13) | 0.002 |
| **Muscle** | 0.66 (0.60 to 1.13) | 0.75 (0.70 to 1.41) | ns |
| **Brain** | 0.19 (0.17 to 0.23) | 0.13 (0.13 to 0.19) | 0.015 |
| **Skin** | 1.81 (1.40 to 2.23) | 2.01 (1.29 to 2.17) | ns |
| **Bone** | 4.09 (3.39 to 4.70) | 2.78 (2.60 to 4.21) | ns |
| **Bone marrow** | 6.44 (6.22 to 7.96) | 9.03 (6.53 to 9.43) | ns |
| **Tumor** | 10.59 (9.66 to 11.95) | 4.68 (4.42 to 5.30) | 0.002 |

Data is presented as median % ID/g values with interquartile range. *P*-values < 0.05 from the Mann-Whitney U-test are shown. LN = lymph node, ns= non-significant.