SUPPLEMENTARY MATERIAL

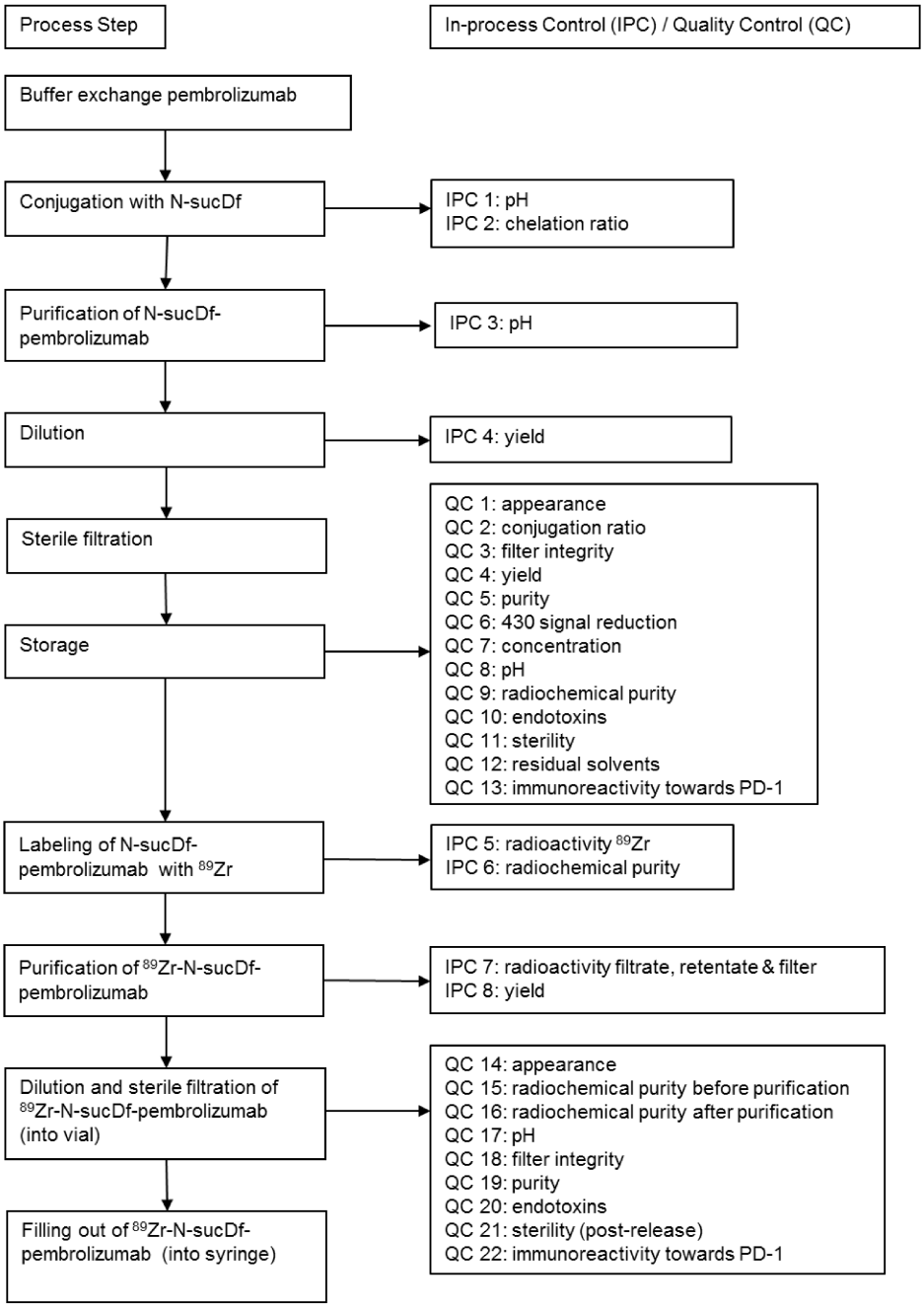

Figure S1: Flow chart of the manufacturing process of the conjugated N-sucDf-pembrolizumab and the ^{89}Zr-pembrolizumab formulation and filling process, including in-process control (IPC) and release quality control (QC) steps.

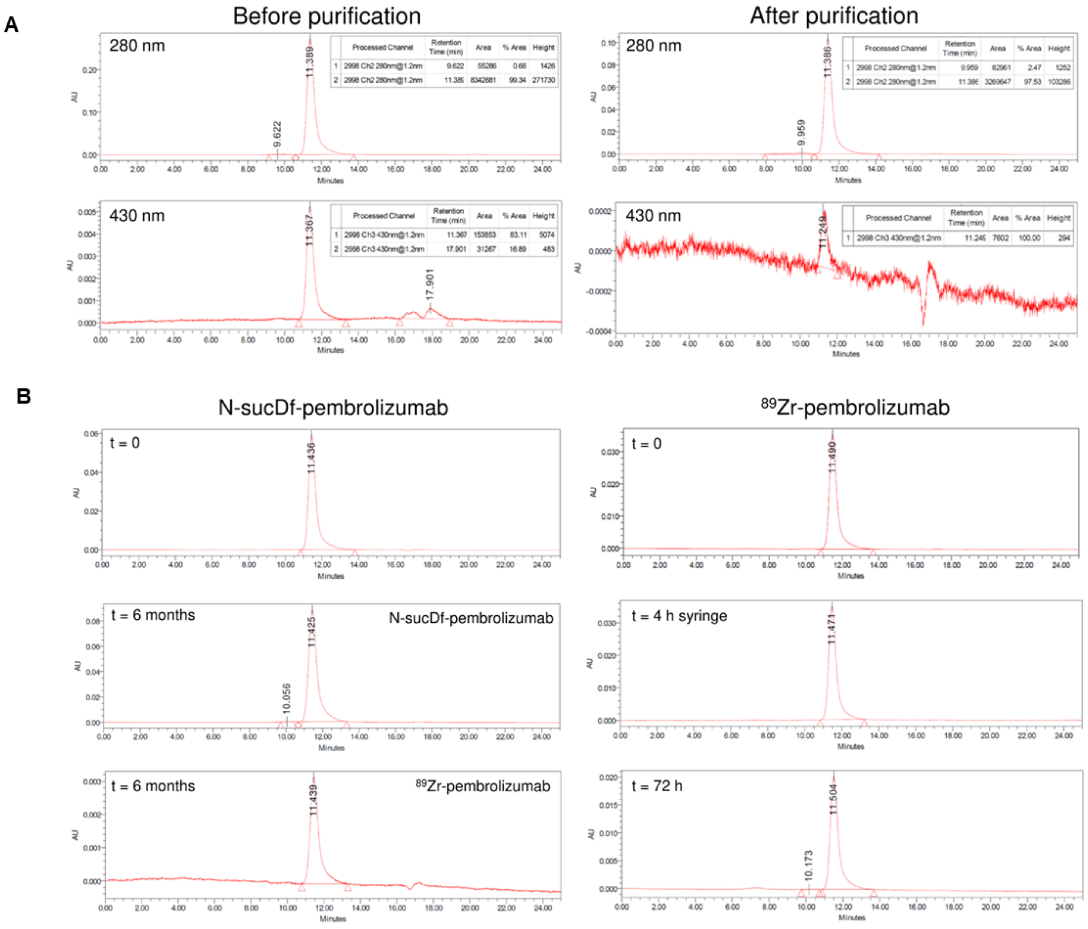

Figure S2: Representative SE-HPLC chromatograms for quality control of N-sucDf-pembrolizumab and ^{89}Zr-pembrolizumab. **A)** SE-HPLC chromatograms for determination of the final number of TFP-N-sucDf ligands per antibody (chelation ratio). Left panel shows chromatograms before purification, right panel after purification. **B)** SE-HPLC chromatograms for determination of stability. Left panels shows chromatograms for stability of N-sucDf-pembrolizumab and ^{89}Zr-pembrolizumab at t = 0 and t = 6 months. Right panel shows chromatograms for stability of ^{89}Zr-pembrolizumab at t = 0, t = 4 h and t = 72 h. Abbreviations: SE-HPLC: size exclusion high-performance liquid chromatography.

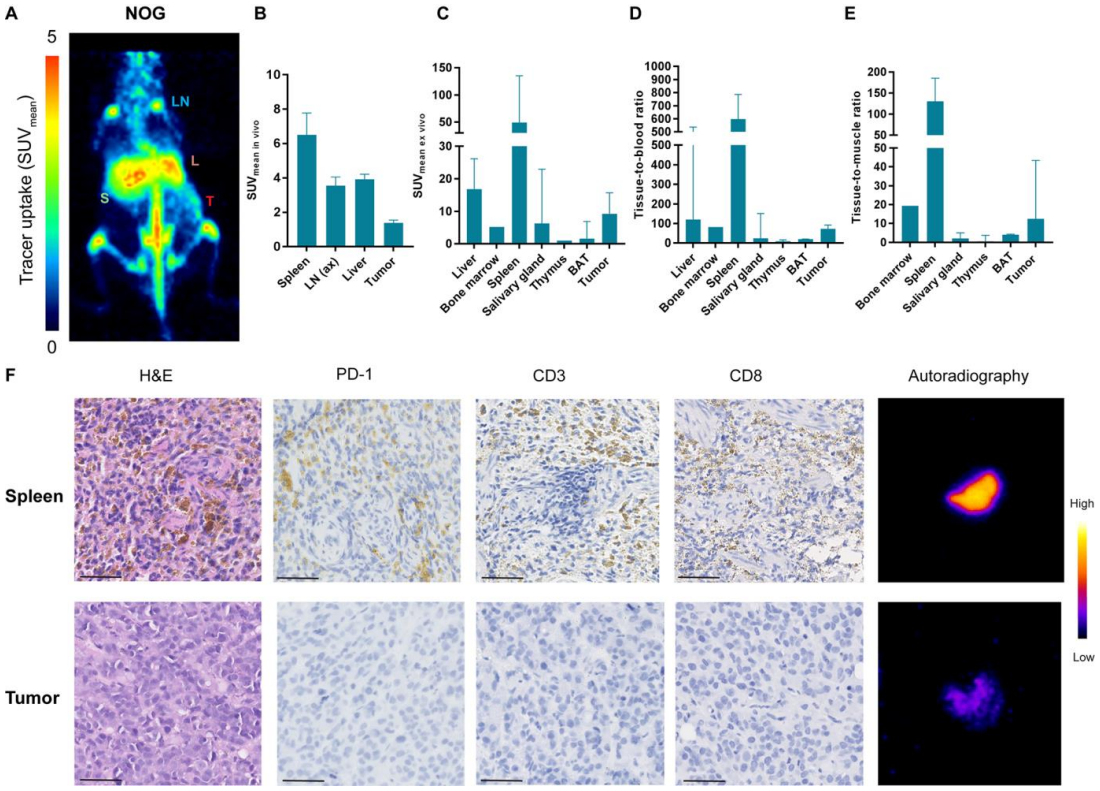

Figure S3: *In vivo* PET imaging and *ex vivo* biodistribution of ^{89}Zr-pembrolizumab in immunodeficient NOG mice. Mice were xenografted with A375M tumor cells and received tracer injection at day 0. PET imaging performed on day 7 pi. **A)** *In vivo* PET example (maximum intensity projection) at day 7 pi showing uptake in tumor (T) uptake, lymph nodes (LN), liver (L) and spleen (S). **B)** *In vivo* uptake of ^{89}Zr-pembrolizumab in liver, spleen, lymph nodes (axillary), liver and tumor, at day 7 pi. Uptake is expressed as SUV_{mean}. **C)** E*x vivo* biodistribution of ^{89}Zr-pembrolizumab in NOG mice. Uptake is expressed as mean radioactivity per gram tissue, adjusted for total body weight ($SUV_{mean\ ex\ vivo}$). **D)** E*x vivo* biodistribution of ^{89}Zr-pembrolizumab in NOG mice, expressed as tumor-to-blood ratio. **E)** E*x vivo* biodistribution of ^{89}Zr-pembrolizumab in NOG mice, expressed as tumor-to-muscle ratio. Data expressed as median ± IQR; *$p \leq 0.05$. Abbreviations: MLN: mesenteric lymph

nodes; BAT: brown adipose tissue. **F)** IHC analysis and autoradiography of spleen and tumor tissue of NOG mice. Formalin-fixed and paraffin embedded (FFPE) tissue blocks where cut into slices of 4 µM and stained for PD-1, CD3 and CD8 (40x). Hematoxylin & eosin (H&E) staining served to analyze tissue viability and morphology (40x). For autoradiography slices were exposed to a phosphor imaging screen for 72 hours and were then scanned using a Cyclone phosphor imager. Scalebar: 50 µm.

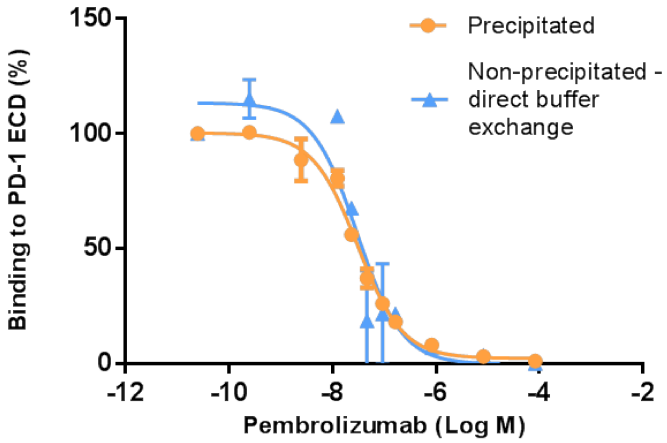

Figure S4: Immunoreactivity assays of different batches of N-sucDf-pembrolizumab. N-sucDf-pembrolizumab, which was precipitated during conjugation (orange line) and N-sucDf-pembrolizumab which was not precipitated during conjugation, by changing pH directly in one step (blue line).

Table S1: GMP manufacturing of N-sucDf-pembrolizumab and ^{89}Zr-pembrolizumab. Batch 1, 2, and 3 fulfill release criteria. In addition, stability data are shown for N-sucDf-pembrolizumab stored at -80°C for 6 months. All release specifications are still met.

Test	Specification	Batch 1	Batch 2	Batch 3	Batch 1 6 M at -80°C
N-sucDf-pembrolizumab					
Appearance	Colorless to light yellow	Colorless	Colorless	Colorless	Colorless
Conjugation ratio of N-SucDf-pembrolizumab	1.5-2.5	1.66	1.55	1.69	Not applicable
Filter integrity	≤ 20%	12%	12%	14%	Not applicable
Yield	> 50%	87.2%	77.8%	78.8%	Not applicable
Impurities of N-SucDf-pembrolizumab	≤ 5%	< 5%	< 5%	< 5%	< 5%
Signal reduction at 430 nm	> 40% reduced	87.4%	79.6%	74.8%	ND according to protocol
Concentration	9.0-11.0 mg/mL	10.13 mg/mL	9.65 mg/mL	10.13 mg/mL	10.83 mg/mL
pH	pH 4.0 - 6.0	4.7	4.9	5.0	4.66
Radiochemical purity (test labeling)	> 95%	99.3%	99.4%	99.3%	99.7%
Endotoxins	≤ 2.5 EU/mL	< 2.5 EU/mL	< 2.5 EU/ml	< 2.5 EU/mL	ND according to protocol
Sterility	Sterile	Sterile	Sterile	Sterile	ND according to protocol
Residual solvents (ACN)	< 410 ppm	< 100 ppm	< 100 ppm	< 100 ppm	ND according to protocol
^{89}Zr-pembrolizumab					
Appearance	Colorless to light yellow	Colorless	Colorless	Colorless	Colorless
Radiochemical purity pre-purification	≥ 70%	97.4%	97.8%	95.5%	97.6%
Radiochemical purity post-purification	≥ 95%	99.3%	99.4%	99.3%	99.7%
pH	pH 5.0-8.0	5.65	5.18	5.66	6.1
Filter integrity	≤ 20%	14%	14%	12%	15%
Impurities of ^{89}Zr-pembrolizumab	≤ 10%	< 10%	< 10%	< 10%	< 10%
Concentration	For information only	0.262 mg/mL	0.094 mg/mL	0.133 mg/mL	0.200 mg/mL
Bacterial endotoxins	≤ 2.5 EU/mL	0.450 EU/mL	0.412 EU/mL	0.532 EU/mL	ND according to protocol
Sterility	Sterile	Sterile	Sterile	Sterile	ND according to protocol
Immunoreactivity towards PD-1	> 70%	202%	88%	114%	231%

Abbreviations: ACN: acetonitrile; ND: not determined; ppm: parts per million

Table S2: Stability data of ^{89}Zr-pembrolizumab. Stability data are shown for two batches ^{89}Zr-pembrolizumab stored 4 h at room temperature (RT) or 168 h at 2-8˚C in the vial. All release specifications are still met.

Test	Specification	Batch 1			Batch 2		
		Original result	4 h RT syringe	168 h 2-8 ˚C vial	Original result	4 h RT syringe	168 h 2-8 ˚C vial
Appearance	Colorless to light yellow	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
pH	pH 4.0 - 6.0	5.65	6.16	6.33	5.40	5.75	6.14
Radiochemical purity (post-purification)	> 95%	99.3%	98.6%	97.1%	99.6%	99.1%	98.2%
Impurities of ^{89}Zr-pembrolizumab	≤ 10%	≤ 10%	≤ 10%	≤ 10%	≤ 10%	≤ 10%	≤ 10%

Table S3: *Ex vivo* biodistribution of ^{89}Zr-pembrolizumab in humanized NOG mice and control NOG mice. Tracer uptake in each organ is expressed as percentage of the injected dose per gram tissue (%ID/g), and as the mean radioactivity per gram tissue, adjusted for total body weight (SUVmean *ex vivo*).

	NOG						huNOG						huNOG block						huNOG IgG_4					
	%ID/g			$SUV_{mean\ ex\ vivo}$			%ID/g			$SUV_{mean\ ex\ vivo}$			%ID/g			$SUV_{mean\ ex\ vivo}$			%ID/g			$SUV_{mean\ ex\ vivol}$		
Tissue	**Med**	**Q1**	**Q3**	**Med**	**Q1**	**Q3**	**Med**	**Q1**	**Q3**	**Med**	**Q1**	**Q3**	**Med**	**Q1**	**Q3**	**Med**	**Q1**	**Q3**	**Med**	**Q1**	**Q3**	**Med**	**Q1**	**Q3**
Blood	**0.3**	0.2	1.4	**0.2**	0.1	0.3	**0.2**	0.2	7.1	**0.1**	0.0	1.8	**9.2**	5.5	35.3	**2.2**	1.4	7.4	**1.8**	0.5	13.7	**0.5**	0.2	4.3
Heart	**11.9**	5.2	14.4	**2.5**	1.0	3.2	**2.7**	2.6	4.3	**0.6**	0.6	1.1	**3.1**	2.2	5.6	**0.8**	0.5	1.2	**2.9**	2.2	5.3	**0.8**	0.5	1.7
Lung	**9.9**	4.2	26.6	**2.2**	0.8	5.6	**2.2**	1.8	3.9	**0.5**	0.4	1	**5.9**	3.0	15.0	**1.5**	0.8	3.2	**2.1**	2.0	6.9	**0.5**	0.5	2.2
Liver	**77.2**	25.4	122.6	**16.9**	5.1	26.2	**25.5**	14.6	39.4	**6.0**	3.4	9.9	**10.7**	8.4	13.5	**2.6**	2.1	2.9	**21.2**	13.4	27.4	**4.9**	3.9	7.1
Kidney	**22.2**	18.8	24.4	**4.5**	3.7	5.6	**5.1**	4.5	9.1	**1.2**	1.1	2.3	**5.4**	4.2	11.4	**1.3**	1.1	2.4	**9.4**	6.5	23.7	**2.4**	1.5	7.3
Stomach	**7.0**	3.4	8.7	**1.5**	0.7	2.0	**2.4**	2.2	4.3	**0.6**	0.5	1.1	**2.2**	1.6	3.4	**0.6**	0.4	0.7	**1.7**	1.3	3.6	**0.4**	0.3	1.1
Pancreas	**4.3**	2.2	12.9	**1.0**	0.5	2.7	**3.1**	2.4	6.2	**0.7**	0.6	1.55	**2.3**	1.6	3.2	**0.6**	0.4	0.7	**2.0**	1.2	5.0	**0.6**	0.3	1.3
Small intestine	**33.4**	17.7	53.3	**7.0**	3.4	12.2	**11.4**	8.1	22.9	**2.7**	1.9	5.7	**6.6**	3.1	8.9	**1.6**	0.8	1.9	**6.3**	4.5	15.8	**1.5**	1.3	4.2
Colon	**5.0**	1.2	11.6	**1.0**	0.2	2.6	**2.1**	1.9	2.9	**0.5**	0.4	0.8	**2.9**	1.3	5.0	**0.7**	0.3	1.2	**2.7**	1.8	4.2	**0.8**	0.5	1.1
Muscle	**6.8**	2.6	22.3	**1.5**	0.5	4.7	**2.8**	2.0	4.3	**0.7**	0.45	1.1	**1.5**	1.1	2.6	**0.4**	0.3	0.6	**2.4**	1.6	3.2	**0.7**	0.4	0.9
Brain	**0.2**	0.1	14.7	**0.0**	0.0	3.1	**0.1**	0.1	1.2	**0**	0	0.4	**0.2**	0.1	0.3	**0.0**	0	0.1	**0.15**	0.1	0.4	**0.1**	0.0	0.1
Bone	**25.6**	14.9	86.7	**4.7**	3.1	20.1	**15.2**	10.1	28.8	**3.6**	2.4	7.25	**6.5**	4.4	9.3	**1.6**	1.1	2.0	**12.7**	12.7	14.8	**3.9**	2.9	4.0
Bone marrow	**28.4**	28.4	28.4	**5.2**	5.2	5.2	**61.9**	25.5	130.2	**14.5**	6.1	32.8	**29.9**	5.3	58.1	**7.1**	1.3	12.4	**32.3**	31.5	33	**8.8**	7.6	10.0
Spleen	**272.5**	78.5	584.3	**49.6**	16.6	135.6	**130.3**	66.5	269.5	**30.5**	15.8	67.7	**22.4**	17.4	30.6	**5.1**	4.3	7.0	**59.8**	26.5	82.2	**13.9**	7.1	21.4
MLN	**-**	-	-	**-**	-	-	**87.2**	35.3	101.1	**20.4**	8.0	25.2	**18.8**	9.6	19.6	**4.1**	2.5	4.6	**10.0**	4.9	17.2	**2.3**	1.4	4.4
Salivary gland	**29.1**	12.1	108.7	**6.3**	2.5	23.0	**11.5**	8.9	18.3	**2.8**	2.1	4.6	**5.7**	5.0	7.8	**1.4**	1.2	1.9	**7.6**	5.1	10.2	**2.1**	1.2	2.9
Thymus	**4.9**	4.2	5.6	**1.0**	1.0	1.0	**5.3**	4.6	8..6	**1.3**	1.1	2.1	**2.7**	2.2	3.5	**0.6**	0.5	0.8	**2.3**	1.5	4.2	**0.5**	0.4	1.1
BAT	**7.6**	6.4	29.9	**1.6**	1.2	6.9	**1.7**	1.5	1.8	**0.4**	0.4	0.4	**1.8**	1.3	2.5	**0.5**	0.3	0.5	**1.5**	1.2	1.8	**0.4**	0.3	0.5
Tumor	**42.5**	22.6	72.6	**9.3**	4.5	15.7	**21.6**	13.5	35.5	**5.1**	3.3	8.9	**18.5**	10.2	33.4	**4.2**	2.5	7.7	**12.7**	11.5	15.8	**3.5**	2.7	4.4