Supporting information

Nanoparticles presenting potent TLR 7/8 agonists enhance anti-PD-L1 immunotherapy in cancer treatment

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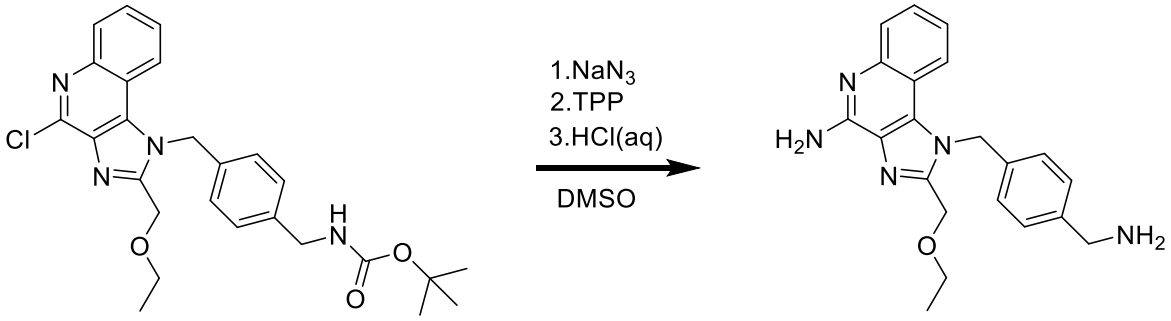
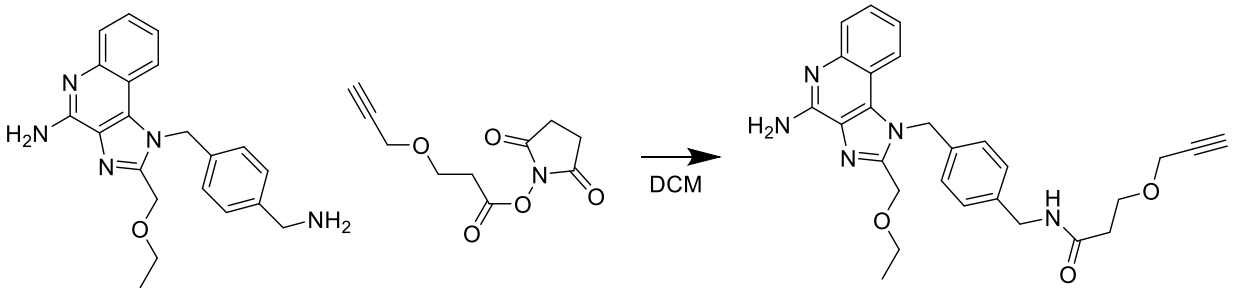
Synthesis of N-(4-((4-amino-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-1-yl)methyl)benzyl)-3-(prop-2-yn-1-yloxy)propenamide (III). The Benzyl amine TLR 7/8 agonist was synthesized as described by Shukla *et. al,*[1] with a modification of the final aromatic substitution and tert-butyl carbamate removal on tert-butyl (4-((4-chloro-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-1-yl)methyl)benzyl)carbamate (**I**), which was done as a one-pot Staudinger-type reaction.[2]



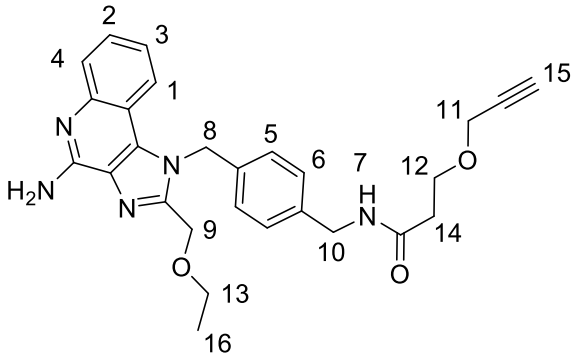
I II

Compound **I** and 2 equivalents of NaN_3 (68 mg, 1.04 mmol) were dissolved in 1.5 mL DMSO, and was heated to 90° C for 2 hours. Triphenylphosphine (2 eq, 273 mg, 1.04 mmol) was added, the temperature was increased to 120° C and the reaction mixture was stirred for 16 hours. 0.5 mL 4M HCl (aq) was added and the temperature was lowered to 95° C for 3 hours. 4 mL of water was added at which a precipitate formed, and the mixture was washed with EtOAc. Na_2CO_3 (sat) was added to the aqueous phase which was extracted with EtOAc. The combined EtOAc was dried with with Na_2SO_4 and the solvent removed *in vacuo* to yield a crude solid. This was purified by silica column chromatography on a biotage isolera system, using a gradient of DCM:MeOH, with 1% trimethylamine in the MeOH. This yielded compound **II** (1-(4-(aminomethyl)benzyl)-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-4-amine) (60 mg, 0.16 mmol, 32% yield).



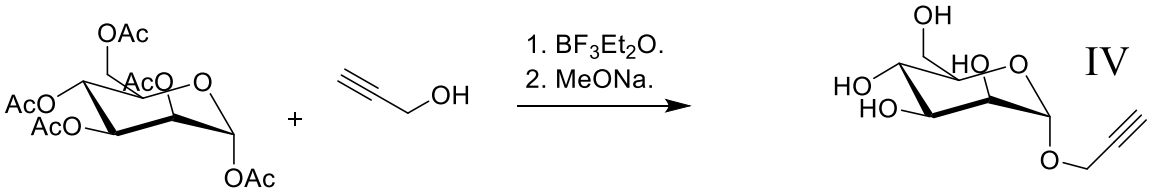
II III

Compound **II** (25 mg, 69 µmol) and propargyl-N-hydroxysuccinimidyl ester (17 mg, 76 µmol) were dissolved in 1 mL anhydrous DCM, and stirred for 2 hours at room temperature. The reaction mixture was loaded onto a silica column, and the product was purified using a DCM:MeOH gradient. This yielded compound **III**, N-(4-((4-amino-2-(ethoxymethyl)-1H-imidazo[4,5-c]quinolin-1-yl)methyl)benzyl)-3-(prop-2-yn-1-yloxy)propanamide (25 mg, 53 µmol, 76% yield).



^{1}H NMR (300 MHz, $CDCl_3$-*d*) δ 8.05 (d, *J* = 8.4 Hz, 1H, H1), 7.73 (d, *J* = 8.3 Hz, 1H, H2), 7.58 (t, *J* = 7.9 Hz, 1H, H3), 7.29 (3H, H4, H5), 7.02 (d, *J* = 7.9 Hz, 2H, H6), 6.43 (s, 1H, H7), 5.91 (s, 2H, H8), 4.78 (s, 2, H9), 4.46 (d, *J* = 5.9 Hz, 2H, H10), 4.15 (d, *J* = 2.4 Hz, 2H, H11), 3.82 (t, *J* = 5.7 Hz, 2H, H12), 3.58 (q, *J* = 7.2 Hz, 2H, H13), 2.55 (t, *J* = 5.7 Hz, 2H, H14), 2.36 (t, *J* = 2.4 Hz, 1H, H15), 1.14 (t, *J* = 7.2 Hz, 3H, H16).

^{13}C NMR (75 MHz, $CDCl_3$) δ 171.35, 166.55, 151.77, 150.14, 138.70, 136.66, 136.09, 133.40, 129.84, 128.43, 125.71, 124.66, 121.24, 120.73, 112.79, 79.06, 75.01, 66.77, 66.02, 64.19, 58.44, 58.38, 58.21, 36.88, 31.89, 14.86. HRMS (ESI) calcd. for $C_{27}H_{29}N_5O_3$+H^+: 472.2343; found 472.2335.



Synthesis of 1'-O-propargyl-α-Mannose (IV). Alkynated mannose was synthesized as described elsewhere.[3] α-Mannose pentaacetate (1.95 g, 5 mmol) and propargyl alcohol (0.34 g, 0.35 mL, 6.3 mmol) were dissolved in dry DCM (44mL) while stirring. BF_3Et_2O (9.3 µL, 8 mg, 0.06 mmol) was added at 0° C, and the mixture was stirred for 16h. The solution was washed with 1M NaOH, and 50 mL EtOAc was added, and the organic phase was washed with brine and dried over Na_2SO_4. The solution was reduced to approximately 5 mL *in vacuo,* and diluted with 5 mL hexanes, and purified by silica chromatography with a EtOAc:Hexanes gradient. The product was dissolved in dry MeOH, and 100 µL 2% NaOMe in MeOH was added. The solution was left for one hour, quenched with a drop of acetic acid, and volatiles removed *in vacuo* to yield **IV**.

General synthesis of N_3-PEG-PLA and MeO-PEG-PLA. PEG-PLA was prepared as previously reported.[4] In short, 0.500 g N_3-$PEG_{(114)}$-OH or MeO-$PEG_{(114)}$-OH in dry DCM (2 mL) with equimolar 1,8-Diazabicyclo[5.4.0]undec-7-ene (15 µL, 15 mg, 0.1 mmol) was rapidly added to a solution of 2.0 g D,L-lactide in 8 mL dry DCM. The mixture was stirred for 8 min, and quenched with the addition of 1 mL acetone with 200 µL acetic acid. The solution was precipitated into 35 mL of a 1:1 mixture of hexanes and diethyl ether in a 50 mL centrifuge tube. The supernatant was discarded, and 35 mL diethyl ether was added to fully precipitate the polymer. This was dissolved in a minimal amount of ethyl acetate, and 35 mL diethyl ether was added to re-precipitate the polymer, 21kDa, Đ=1.16 by PEG standards. ^{1}H-NMR showed a PLA block of 20.5 kDa DP=287, calculated from PEG signals (5kDa, DP=114).

General synthesis of TLR7/8a-PEG-PLA and Mannose-PEG-PLA conjugates. Conjugations were performed analogous to the procedure previously reported.[4] A 20 mL scintillation vial was charged with **III** or **IV** (1.5 eq) and azido poly(ethylene oxide)-*b*-poly(D,L-lactide) 5kDa-20kDa (0.5 g, 20 µmol) was dissolved in 4 mL of NMP and sparged with nitrogen for 10 min. 0.1 mL of a degassed solution of CuBr (3.7 mg/mL) and THPTA (16 mg/mL) was added. The reaction mixture was further sparged with nitrogen for 10 min. The reaction mixture is incubated for 16h at room temperature and precipitated into diethyl ether in a 50 mL centrifuge tube to recover the polymer. The polymer was then dissolved in ethyl acetate and precipitated into diethyl ether, and dried in vacuo.

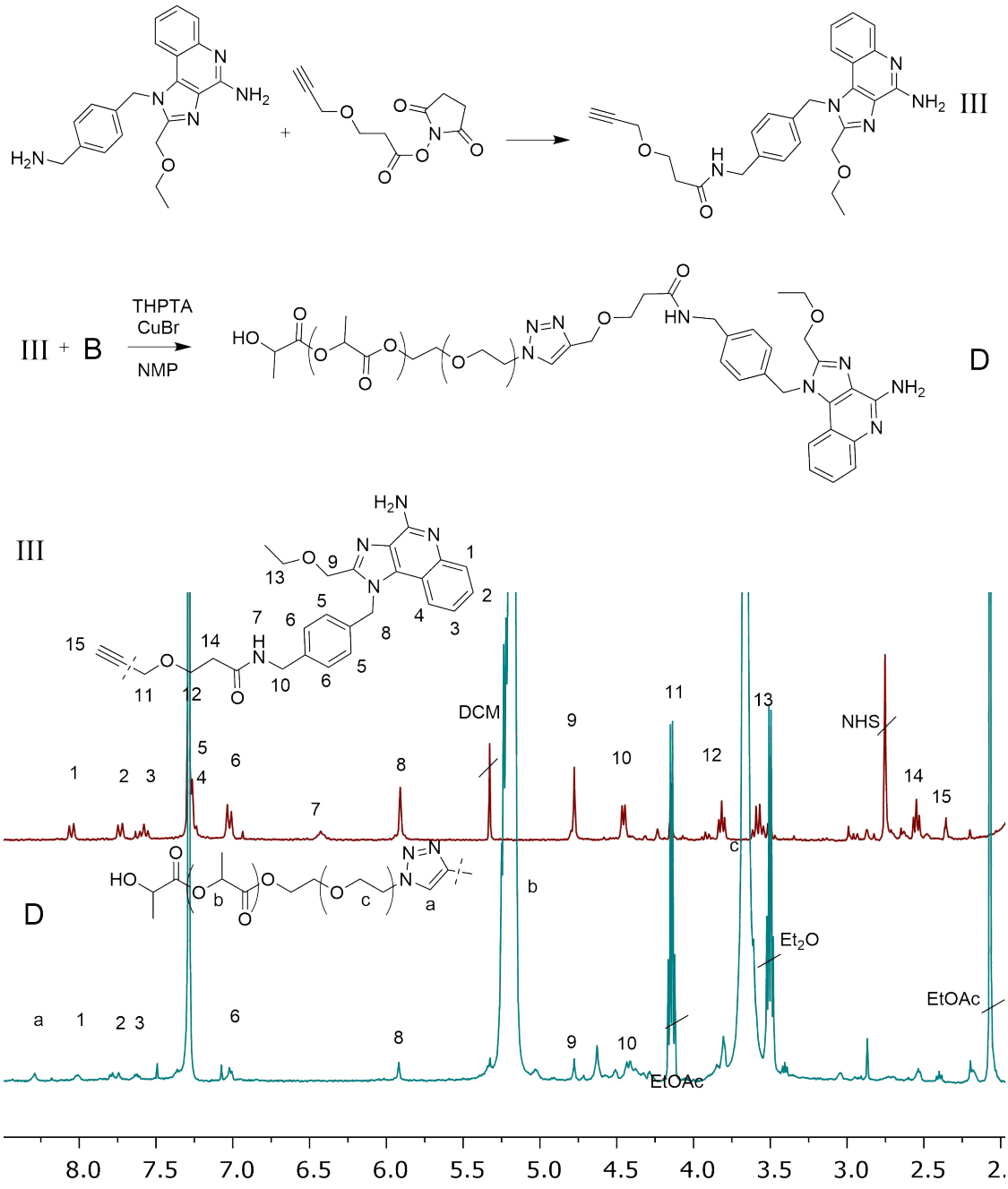


Figure S1: Stacked ^{1}H-NMR ($CDCl_3$) of **III** and TLR7/8a-PEG-PLA conjugate.

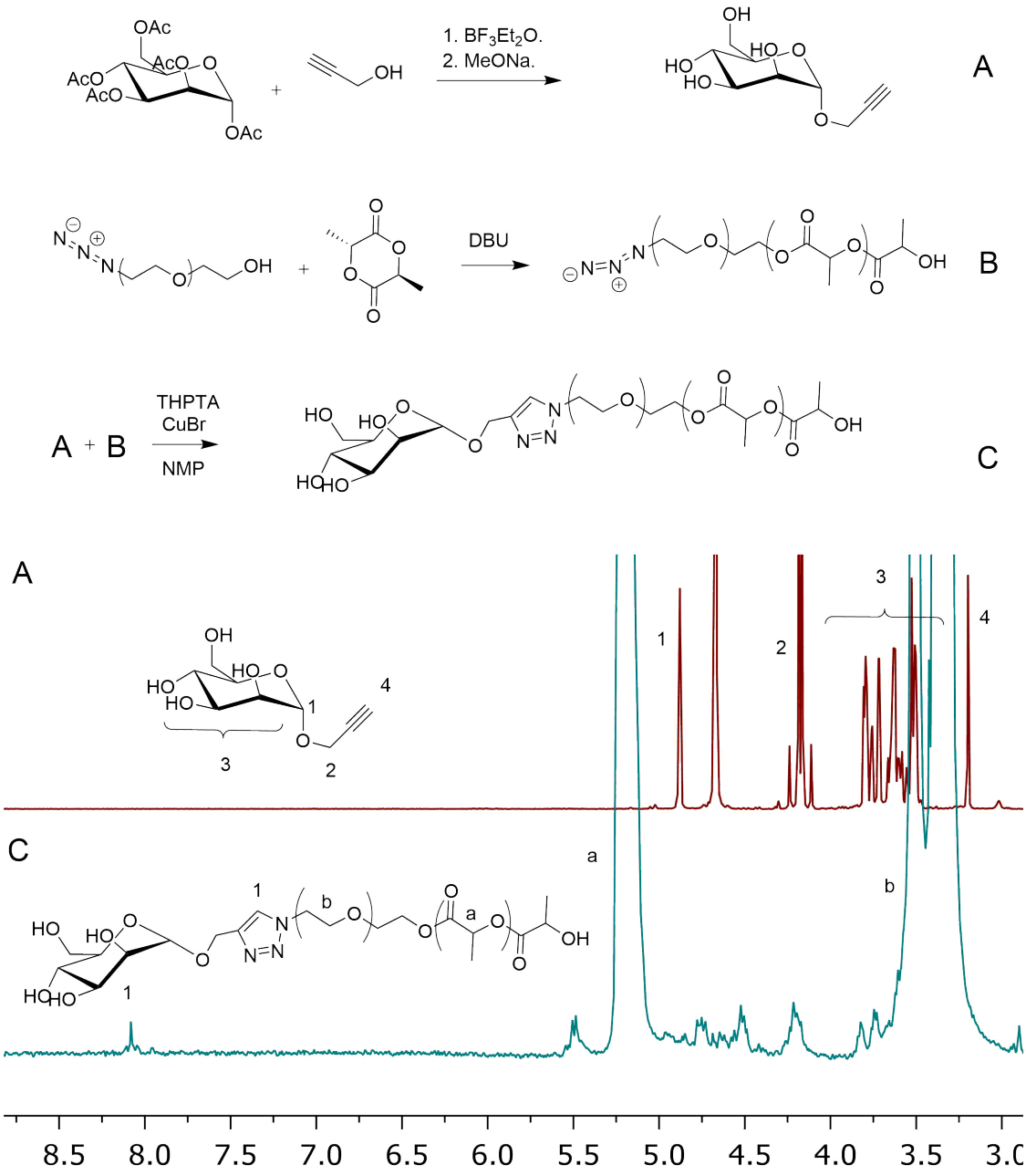


Figure S2: Stacked ^{1}H-NMR of **IV** (D_2O) and Mannose-PEG-PLA conjugate (d6-DMSO).

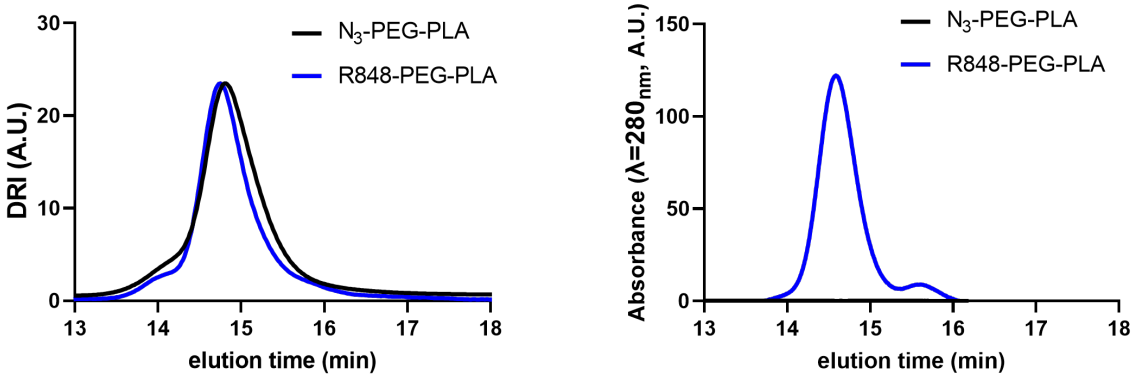


Figure S3: TLR7/8a conjugation to PEG-PLA confirmed by SEC. UV-SEC traces of N_3-PEG-PLA and TLR7/8-PEG-PLA was compared with UV absorbance normalized to the RI peak intensities. TLR7/8-PEG-PLA shows strong UV absorption at 280 nm, with no absorption from N_3-PEG-PLA.

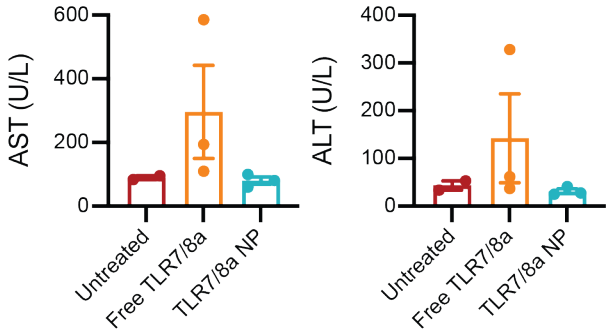


Figure S4: Liver enzymes AST and ALT in serum from mice treated 4 times with either free TLR7/8a or TLR7/8a NPs (n=3). Treatment with TLR7/8a NPs led to similarly low levels of both AST and ALT as was seen in serum samples from the control untreated mice (n=2). Treatment with free TLR7/8a led to higher and more varied quantities of liver enzymes compared to the control.

Table S1. Composition of different nanoparticles tested.

NP Treatment	PEG-PLA (%)	TLR7/8a-PEG-PLA (%)	Mannose-PEGPLA (%)
High Valency NP	50	50	
Medium Valency NP	75	25	
Low Valency NP	90	10	
High Mannose	30	20	50
Low Mannose	60	20	20
High Valency/High Mannose		50	50
Medium Valency/High Mannose	25	25	50

Table S2. NP size by composition and precipitation solvent

Ratio TLR7/8a - PEGPLA/PEGPLA	Diameter (nm)	S	PDI	S
50/50	39.2	0.4	1.51E-01	4.43E-02
25/75	34.9	0.4	7.81E-02	2.81E-02
10/90	33.1	0.4	8.62E-02	1.90E-02
0/100	31.1	0.3	5.46E-02	1.52E-01

100% AcCN

Ratio TLR7/8a - PEGPLA/PEGPLA	Diameter (nm)	S	PDI	S
50/50	38.9	0.3	1.43E-01	2.96E-02
25/75	34.8	0.4	8.80E-02	4.08E-02
10/90	32.7	0.3	6.97E-02	3.50E-02

1:2 DMSO:AcCN

NP type	Diameter (nm)	S	PDI	S
High Mannose	38.6	0.3	8.47E-02	2.19E-02
Med Mannose	51.5	0.4	1.64E-01	2.34E-02
Low Mannose	43.5	0.2	9.27E-02	3.23E-04

Table S3. NP size and PDI by DLS

Ratio TLR7/8a - PEGPLA/PEGPLA	**Diameter (nm)**	**PDI**
50/50	39.5	1.36E-01
25/75	33.7	1.07E-01
10/90	31.7	5.24E-02
0/100	30.3	8.56E-02

Ratio TLR7/8a - PEGPLA/PEGPLA	**Zeta Potential (mV)**	**Std. Dev.**
10/90 NP	-14.8	0.8
50/50 NP	-14.2	0.6
50/50 NP (Mannose-PEGPLA)	-9.8	0.4
MeOPEGPLA NP	-28	1

Table S4. MFI Luminex values and corresponding P-values

Cytokine	NP 1	NP 2	NP 3	Sol. 1	Sol. 2	Sol. 3	p-value	FDR Correction[#] (q-value)	Bonferroni Correction[&] (p-value)
TGFβ	1119	516.5	657.75	1548.25	1063.75	945.5	0.1791	0.8737	>0.9999
IL10	51	49.5	48.5	79.5	116.5	84.75	0.0645	0.8737	>0.9999
VEGF	79	60.75	68.5	86.25	96.75	91.25	**0.0226**	0.8737	>0.9999
LIF	38.25	40.75	49.75	54.75	54.25	72.5	0.0645	0.8737	>0.9999
IL6	93.25	182.25	110.5	1548.5	2797	1463	**0.0139**	**0.0144**	0.0984
IFNα	67.25	43.75	56.75	1287.25	2719.75	770.5	0.0579	**0.0480**	0.3817
IL9	57	42.5	59.75	85.75	78.75	406	0.2734	0.8737	>0.9999
IL12P70	58	58	58.75	164.5	244.5	160.25	**0.0087**	0.8737	>0.9999
IL15/IL15R	38	44.5	36.5	92	136	92	**0.0108**	0.8737	>0.9999
IFNγ	39	47	37.25	76.5	100	77.5	**0.0061**	0.8737	>0.9999
GSCF/CSF3	72.25	63	46	76.5	116.25	87.5	0.0818	0.8737	>0.9999
IL22	37.25	33	30.25	56.75	75	60	**0.0070**	0.8737	>0.9999
GMCSF	29.75	33	31.5	64.75	99	71	**0.0114**	0.8737	>0.9999
IL27	28.5	32.25	28	34.5	40.75	514.75	0.3527	0.8737	>0.9999
IL3	27.25	34.5	30.5	30	39	38.5	0.2303	0.8737	>0.9999
IL4	38.5	44	42	55	66.75	59.75	**0.0073**	0.8737	>0.9999
MCSF	45.5	53	48	56.25	55.5	66.25	0.0629	0.8737	>0.9999
IL5	98	107.75	53.75	204.25	232.75	198.25	**0.0032**	0.8737	>0.9999
IL2	187.5	261.25	291.75	178.75	284.75	188.75	0.5558	0.8737	>0.9999
MIP1B	367.25	623.75	131.25	11759.25	16535.75	8211.25	**0.0081**	**<0.0001**	**<0.0001**
MCP3	2518	2288.5	3956	12351.75	12621.25	11008.25	**0.0002**	**<0.0001**	**<0.0001**
IP10	974	1001.5	830.5	9204.5	8625.25	7510	**0.0001**	**<0.0001**	**<0.0001**
MCP1	138.25	453.5	134.5	8219.25	13884.5	7932	**0.0073**	**<0.0001**	**<0.0001**
GROA	127.5	511.5	662.25	3052	2490.5	1847.75	**0.0061**	**0.0051**	**0.0287**
EOTAXIN	3291.5	1456	5327.75	5348.5	3325.5	4960.75	0.4059	0.1898	>0.9999
MIP1A	87.5	132.5	85.25	798	1423.25	629	**0.0248**	0.4998	>0.9999
RANTES	309.5	261.5	307.25	1244	1706.25	898.75	**0.0134**	0.3478	>0.9999
MIP2	120.5	107.75	113.5	150	141.5	132.25	**0.0124**	0.8737	>0.9999
LIX	240	272.25	407.5	773.75	612.5	802.25	**0.0057**	0.8737	>0.9999
TNFα	71	150.5	79.5	738.5	1005.75	469	**0.0153**	0.8370	>0.9999
IL18	47.75	67	50.75	189.5	303	205.75	**0.0078**	0.8737	>0.9999
IL17A	27.75	35	24.5	158.25	254.25	116	**0.0231**	0.8737	>0.9999
IL13	46	47.75	51.25	107.5	111.5	91	**0.0010**	0.8737	>0.9999
IL31	35	49.5	38.25	191	309.5	168	**0.0145**	0.8737	>0.9999
IL1A	45	48	44.75	65.5	77	64	0.0057	0.8737	>0.9999
IL23	38.5	35	33.5	48	52.5	52	**0.0018**	0.8737	>0.9999
IL1B	25.5	24.25	21.25	35.5	42.5	39.75	**0.0029**	0.8737	>0.9999

[#]Two-stage step-up method of Benjamini, Krieger and Yekutieli on Prism

[&]Multiple comparisons with Bonferroni method on Prism

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