Depletion of CD206^{+} tumour macrophages by mUNO targeted nanoconjugate inhibits tumourigenesis and dissemination in triple negative breast cancer

Anni Lepland[¥,1], Alessio Malfanti[¥,2], Uku Haljasorg[3], Eliana K. Asciutto[4], Monica Pickholz[5,6], Mauro Bringas[7,8], Snežana Đorđević[2], Liis Salumäe[9], Pärt Peterson[3], Tambet Teesalu[ψ,1,10], María J. Vicent[ψ,2], Pablo Scodeller[ψ,1]

¥: equal contribution
ψ: equal contribution

[1] Laboratory of Precision and Nanomedicine, Institute of Biomedicine and Translational Medicine, University of Tartu, Ravila 14B, Tartu, 50411, Estonia

[2] Polymer Therapeutics Laboratory, Prince Felipe Research Center, Av. Eduardo Primo Yúfera 3, Valencia 46012, Spain

[3] Molecular Pathology Research Group, Institute of Biomedicine and Translational Medicine, University of Tartu, Ravila 19, Tartu, 50412, Estonia

[4] School of Science and Technology, National University of San Martin (UNSAM) ICIFI and CONICET. Buenos Aires, Argentina.

[5] Departamento de Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires

[6] Instituto de Física de Buenos Aires (IFIBA), CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina

[7] Departamento de Química Inorgánica, Analítica y Química Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad de Buenos Aires, C1428EHA, Argentina

[8] Fundación Instituto Leloir, Instituto de Investigaciones Bioquímicas de Buenos Aires (IIBBA-CONICET), C1405BWE Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina.

[9] Tartu University Hospital, Pathology department, Puusepa 8, 50406, Estonia

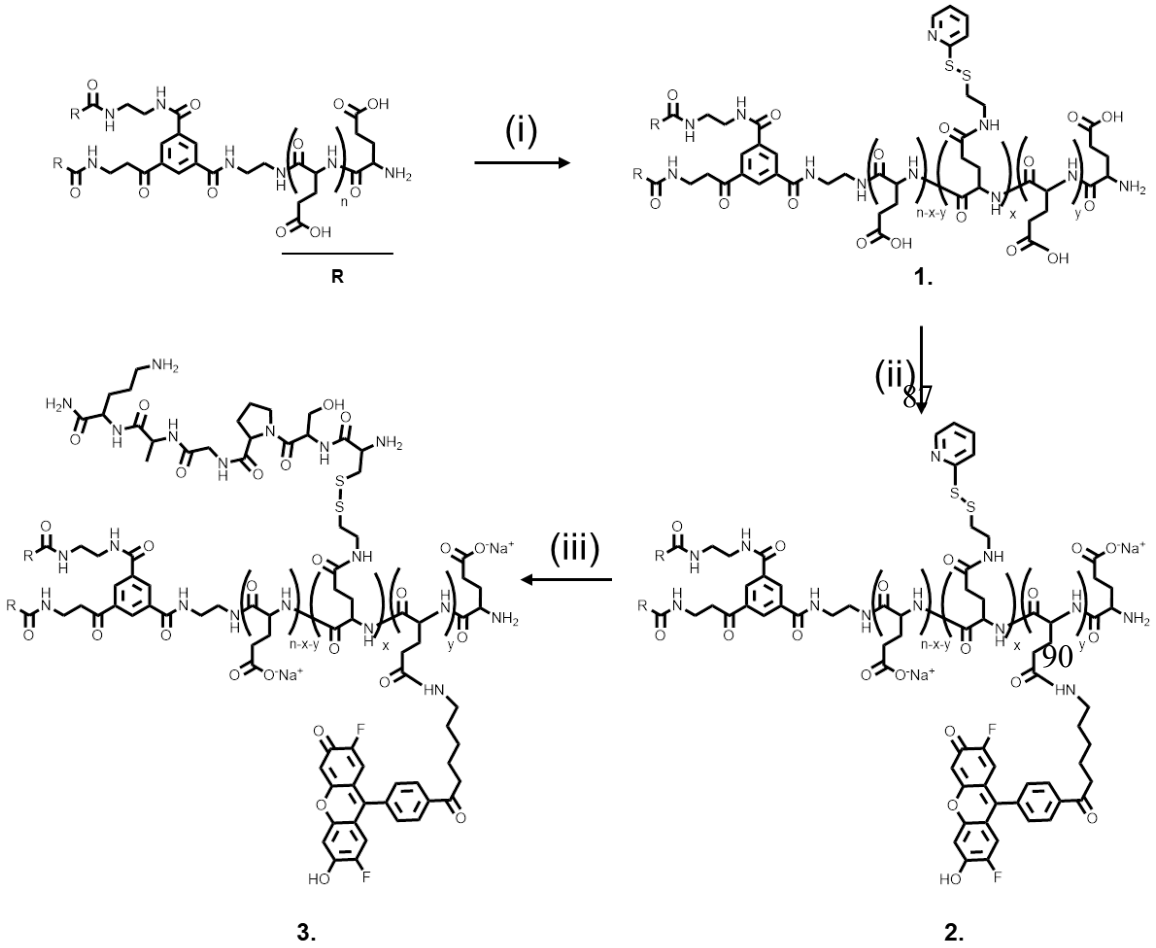
[10] Centre for Nanomedicine and Department of Cell, Molecular and Developmental Biology, University of California, Santa Barbara, 93106, CA, USA

Synthetic procedures

Materials

Three arms benzenetricarboxamide (BTA) centred star-shaped poly-L-glutamates (St-PGA) was kindly provided by Polypeptide Therapeutic Solution. The mUNO peptide (CSPGAK-COOH) was purchased from TAG Copenhagen. The Oregon Green™ 488 (OG) Cadaverine and trifluoroacetic acid were purchased from Thermofisher Scientific. Doxorubicin hydrochloride salt (DOX) was purchased from Xingcheng Chempharm Co. Ltd. Daunorubicin HCl (DAU), cathepsin B from bovine spleen, dithiothreitol, and sodium acetate were purchased from Sigma-Aldrich and used without further purification unless otherwise indicated. Deuterium oxide was purchased from Deutero GmbH. Size exclusion chromatography (SEC) was performed using Sephadex® LH-20, and columns were purchased from GE Healthcare Bio-Sciences AB. Dialysis was performed in a Millipore ultrafiltration device fitted with a 3 kDa molecular weight cut-off (MWCO) regenerated cellulose membrane (Vivaspin®, Merck). For the drug release study, $EDTANa_2$, PBS, chloroform, liquid-chromatography–mass spectrometry (LC-MS) grade methanol, and water were purchased from Merck, while LC-MS grade acetonitrile was purchased from Fisher chemical.

Synthesis of St-PGA-OG-mUNO



Scheme S1. Synthetic approach to obtain St-PGA-OG-mUNO nanoconjugate. (i) *a.* DMTMM BF_4, 30 min, RT, anh-DMF, *b.* pyridyldithiol (PD) amine, DIEA, pH 8, anh-DMF, 24 h; **(ii)** *a.* DMTMM BF_4, 30, RT, anh-DMF, *b.* OG_{488}-cadaverine, DIEA, pH 8, anh-DMF, 24 h; **(iii)** mUNO, 3 h, PBS, pH 7.4.

Synthesis of St-PGA-PD

The synthesis of St-PGA-PD (PD – pyridyldithiol) was performed according to previously published protocols[1,2]. St-PGA (50 mg, 0.387 mmol glutamic acid units (GAU), 1 eq.) was dissolved in 5 mL of anhydrous N,N'-dimethylformamide (DMF) under nitrogen flow. Then, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM BF_4, 9.06 mg, 0.075 eq.) was added after dissolving it in anhydrous DMF. The solution was stirred for 30 min at RT. Then, pyridyldithiol amine (4.1 mg, 0.06 eq.) was added to the solution and the pH was adjusted to 8 by adding N,N-diisopropylethylamine (DIEA). The mixture was kept under magnetic stirring for 24 h at RT. The mixture was purified by precipitation in diethyl ether (3x100 mL). The compound was further purified through either acid/base precipitation. A white amorphous solid was obtained after freeze-drying.

Yield: 80-90%.

1H NMR δH (300MHz, D_2O): 8.53-8.19 (1H, b), 7.93-7.74 (2H, b), 7.45-7.23 (1H, b), 4.48-4.23 (1H, b), 2.40-1.79 (4H, m).

Synthesis of St-PGA-PD-OG

The synthesis of St-PGA-PD-OG was performed according to a Van Lysebetten et al. protocol[2]. St-PGA-PD (25 mg, 0.1935 mmol GAU, 1 eq.) was dissolved in 2.5 mL of anhydrous DMF under nitrogen flow. Then, DMTMM BF_4 (4.53 mg, 0.075 eq.) dissolved in anhydrous DMF was added. The solution was stirred for 30 min at RT. Then, OG_{488}-cadaverine (1.1 mg, 0.012 eq.) was added to the solution, and the pH was adjusted to 8 by adding DIEA. The mixture was kept under magnetic stirring for 24 h at RT and protected from the light. The mixture was purified by precipitation in diethyl ether (3x100 mL). The fine powder obtained was dissolved in DMF and passed twice through an LH-20 column. The first eluting fraction, corresponding to the acid form of the St-PGA-PD-OG, was collected and dried under vacuum, and the water-soluble sodium salt form of the final product was obtained by dissolving the resulting solid in 0.1 M $NaHCO_3$. The excess of the free drug was further removed by using Vivaspin® 3 kDa. An orange-red amorphous solid was obtained after freeze-drying.

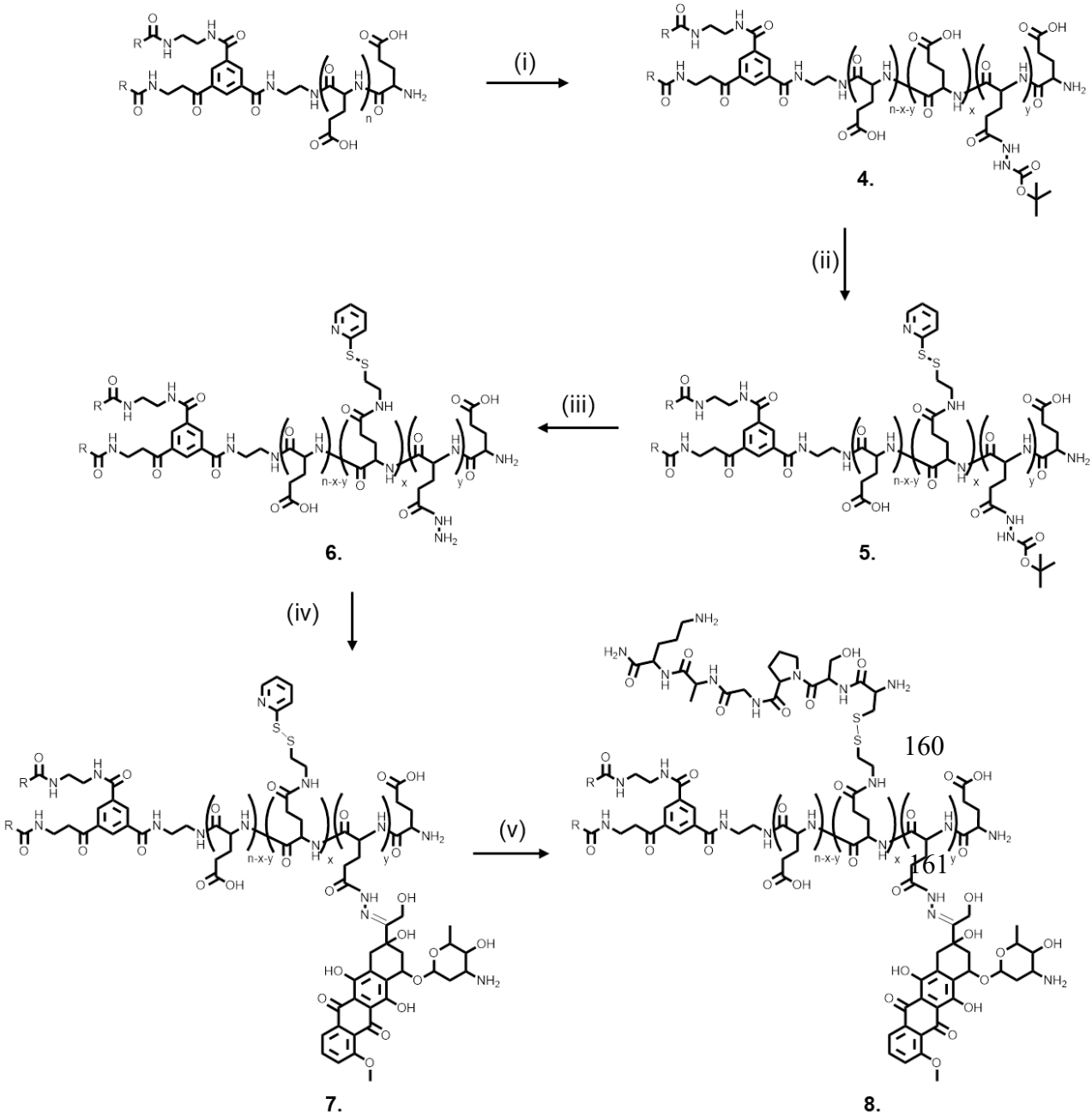
Yield: 75-80% wt.

Synthesis St-PGA-OG-mUNO

The synthesis of St-PGA-OG-mUNO was performed as described according to a set-up protocol[2]. Briefly, St-PGA-PD-OG (20 mg, 1.0 eq) was dissolved at the concentration of 10 mg/mL in PBS, pH 7.4, RT under gently stirring. The mUNO peptide (4.1 mg, 0.055 eq.) was dissolved in PBS, immediately added to the solution, and stirred for 3 h in the dark. Then, the reaction was purified by Vivaspin® 3 kDa. The final product was lyophilised, and an amorphous orange-red solid was obtained.

Yield: 60% wt.

Synthesis of St-PGA-DOX-mUNO ("OximUNO")



Scheme S2. Synthetic approach to achieve OximUNO conjugate. (i) *a.* DMTMM BF_4, 30, RT, anh-DMF, *b.* tert-butylcarbazate, DIEA, pH 8, anh-DMF, 24 h; **(ii)** *a.* DMTMM BF_4, 30, RT, anh-DMF, *b.* pyridyldithiol amine, DIEA, pH 8, anh-DMF, 24 h; **(iii)** TFA, 30'; **(iv)** DOX, anh-DMF, CH_3COOH (cat), pH 5, 72 h; **(v)** mUNO, 3 h, PBS, pH 7.4.

Synthesis of St-PGA-Hz-Boc (fourth step)

The synthesis of St-PGA-Hz-Boc was performed according to a protocol slightly modified from Arroyo-Crespo et al.[3]. Briefly, St-PGA (500 mg, 3.67 mol GAU, 1 eq.) was dissolved in 10 mL of anhydrous DMF under nitrogen flow. Afterward, DMTMM BF_4 (90.56 mg, 0.075

eq.) dissolved in anhydrous DMF was added. The solution was stirred for 30 min at RT. Then, tert-butylcarbazate (29.15 mg, 0.06 eq.) was added to the solution, and the pH was adjusted to 8 by adding DIEA. The mixture was kept under magnetic stirring for 24 h at RT. The mixture was purified by precipitation in diethyl ether (3x300 mL). The compound was further purified through either acid/base precipitation. A white amorphous solid was obtained after freeze-drying.

Yield: 80-90% wt.

1H NMR δH (300MHz, D_2O): 4.36-4.14 (1H, b), 2.40-1.79 (4H, m), 1.42-1.37 (9H, s).

Synthesis of St-PGA- Hz-Boc-PD (fifth step)

St-PGA (250 mg, 1.935 mol GAU, 1 eq.) was dissolved in 10 mL of anhydrous DMF under nitrogen flow. Then, DMTMM BF_4 (45.28 mg, 0.075 eq.) dissolved in anhydrous DMF was added. The solution was stirred for 30 min at RT. Then, pyridyldithiol amine (20.46 mg, 0.06 eq.) was added to the solution and the pH was adjusted to 8 by adding DIEA. The mixture was kept under magnetic stirring for 24 h at RT. The mixture was purified by precipitation in diethyl ether (3x300 mL). The compound was further purified through either acid/base precipitation. A white amorphous solid was obtained after freeze-drying.

Yield: 80-90% wt.

1H NMR δH (300MHz, D_2O): 8.49-8.28 (1H, b), 7.95-7.81 (2H, b), 7.45-7.28 (1H, b), 4.49-4.19 (1H, b), 2.41-1.81 (4H, m), 1.57-1.42 (9H, s).

Deprotection of St-PGA-Hz and St-PGA- Hz-PD (sixth step)

The deprotection of protected St-PGA-Hz-Boc and St-PGA -Hz-Boc-PD (200 mg each) was performed by dissolving the conjugates in trifluoroacetic acid (100%, 5 mL). After complete dissolution, the reaction was allowed to continue for 30 minutes. The nanoconjugates were precipitated in diethyl ether (3x300 mL), washed with acidic water, and lyophilised.

Yield: 70% wt.

1H NMR δH (300MHz, D_2O): 8.51-8.27 (2H, b), 7.90-7.79 (1H, b), 7.40-7.27 (2H, b), 4.50-4.22 (1H, b), 2.47-1.91 (4H, m).

Synthesis of St-PGA-DOX and St-PGA- DOX-PD (seventh step)

Deprotected polymers were conjugated with DOX following a previously published protocol by Arroyo-Crespo et al.[3] Briefly, St-PGA-Hz or St-PGA-Hz-PD (100 mg, 0.734 mmol, 1 eq.) and DOX (42.57 mg, 0.1 eq.) were dissolved in 5 mL of anhydrous DMF at RT under magnetic

stirring. After the full solubilisation of reagents, acetic acid (glacial, 100 µL) was added to the solution to reach pH 5. The reaction was allowed to proceed for 72 h at RT, protected from the light. After that time, the reaction volume was reduced by half, and the mixture was purified by passing it through an LH-20 column two times to remove the unreacted DOX. The first eluting fraction, corresponding to the acid form of the DOX-conjugate, was collected and dried under vacuum and the water-soluble sodium salt form of the final product was obtained by dissolving the resulting solid in 0.1 M $NaHCO_3$. The excess of the free drug was further removed using Vivaspin® 3 kDa. The final product was lyophilised, and an amorphous dark red solid was obtained.

Yield(s):

- St-PGA-DOX: 70-80 % wt
- St-PGA-DOX-PD: 70 % wt

Synthesis of OximUNO (eighth step)

St-PGA-PD-DOX (80 mg, 1.0 eq) was dissolved at the concentration of 10 mg/mL in PBS, pH, 7.4, RT under gentle stirring. The mUNO peptide (16.35 mg, 0.055 eq.) was dissolved in PBS, immediately added to the solution, and stirred for 3 h in the dark. Then, the product was purified by Vivaspin® 3 kDa. The final product was lyophilised, and an amorphous dark red solid was obtained.

Yield: 60% wt.

OG/DOX/mUNO loading determination

OG loading was determined by fluorescence spectroscopy (λex= 501 nm, λem= 526 nm) using a calibration curve previously made with the OG standard solutions in the concentration range 0.5-20 µg/mL ($y=6\cdot10^6x - 7240$, $R^2= 0.9988$).

DOX loading was determined by UV-VIS at 480 nm using a calibration curve previously made with DOX standard solutions in the concentration range of 5.0-50 µg/mL ($y=0.0197x + 0.0045$, $R^2= 0.9988$).

mUNO loading was estimated by ^{1}H-NMR and further confirmed by LC-MS amino acid analysis performed at the University of Barcelona (Unitat de Tècniques Separatives I Síntesi de Pèptids Centres Cientifics I Tecnològics).

Free drug determination

OximUNO nanoconjugate (3 mg/mL) was suspended in 500 µL of methanol (LC-MS grade) with DAU as internal standard, vortexed for 2 min and centrifuged at 30 437g for 10 min. The supernatant was filtered through a 0.45 µm filter and subjected to the LC-MS analysis.

Supplementary figures and videos

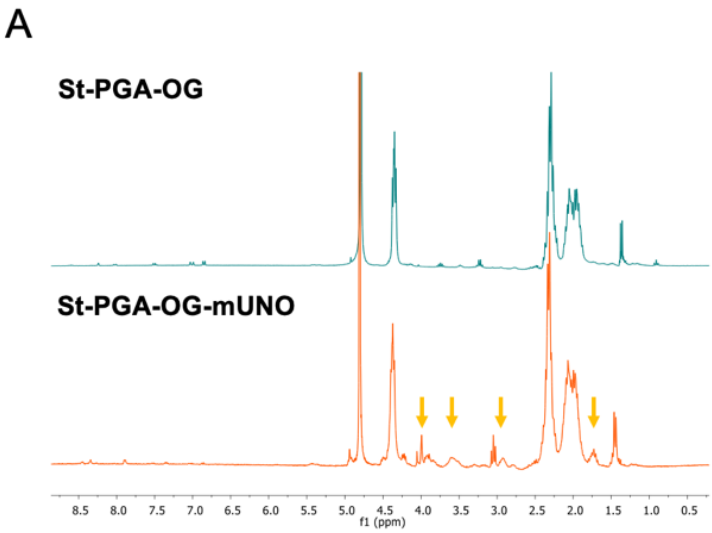


Fig. S1. Representative characterisation of St-PGA-OG and St-PGA-OG-mUNO. (**A**) ^{1}H-NMR in D_2O of the conjugates. The orange arrows show the peaks from mUNO.

Video S1. 50 ns MD trajectory of St-PGA in solution. Water and ions were removed for visualization purposes. PGA chains are shown in white by overlaying in magenta the regions that form the alpha-helix structure, and the BTA core is shown in green.

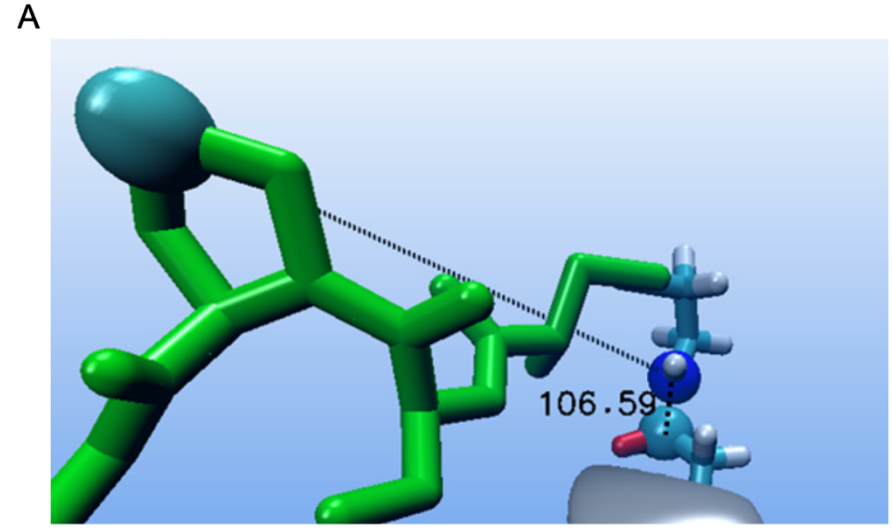


Fig. S2. The angle used to characterise the rotation of mUNO around the PGA. (**A**) Angle formed by an aromatic carbon of mUNO's proline (green sphere), a nitrogen of the pyridyldithiol linker (blue sphere) and an aromatic carbon of the glutamic acid (light blue sphere).

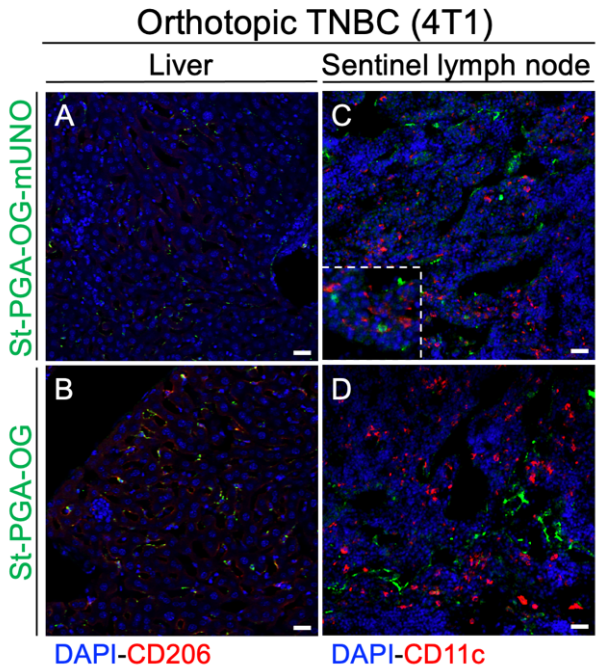


Fig. S3. St-PGA-OG-mUNO shows low accumulation in the liver in the orthotopic TNBC model. St-PGA-OG-mUNO (0.41mg/0.5mL) or St-PGA-OG (0.35mg/mL) was injected i.p. 10 days post tumour induction (s.c. injection of $1x10^6$ 4T1 cells), N=3. Nanoconjugates were circulated for 6 h, after which mice were sacrificed and organs collected for analysis. (**A, B**) Both St-PGA-OG-mUNO and St-PGA-OG showed low accumulation in the liver. (**C, D**) Both conjugates showed no colocalisation with $CD11c^+$ dendritic cells in the sentinel lymph node. Scale bars represent 20 μm.

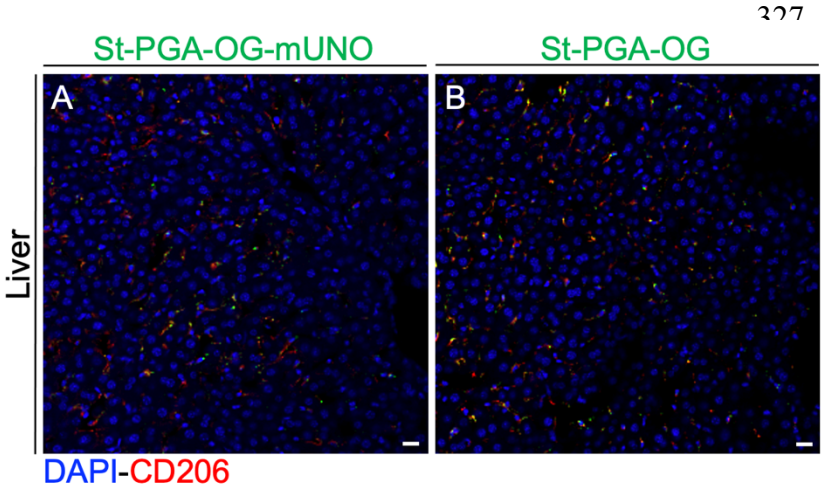


Fig. S4. St-PGA-OG-mUNO shows low accumulation in the liver with the experimental metastases model of TNBC. St-PGA-OG-mUNO (0.41mg/0.5mL) or St-PGA-OG (0.35mg/0.5mL) was injected i.p. 10 days post tumour induction in lungs (i.v. injection of $5x10^5$ 4T1 cells), N=2. Nanoconjugates were circulated for 6 h, after which mice were sacrificed and organs collected for analysis. (**A**) St-PGA-OG-mUNO displayed low accumulation in the liver. (**B**) The same was seen with St-PGA-OG. Scale bars represent 20 µm.

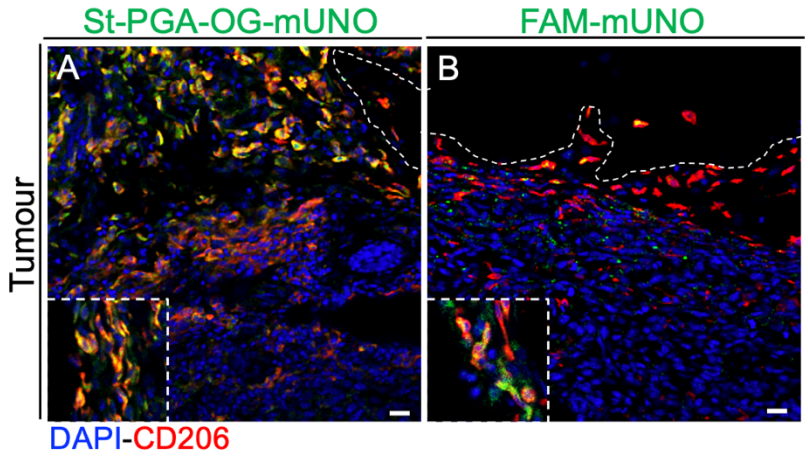


Fig. S5. St-PGA-OG-mUNO shows higher colocalisation compared to FAM-mUNO. St-PGA-OG-mUNO (30nmoles in OG) or FAM-mUNO (30nmoles in FAM) was injected i.p. 10 days post tumour induction (s.c. injection of $1x10^6$ 4T1 cells), N=2. The nanoconjugate or free peptide was circulated for 6 h, after which mice were sacrificed and organs collected for analysis. (**A**) St-PGA-OG-mUNO showed higher OG/CD206 colocalisation than FAM-mUNO (**B**), indicating that multivalent presentation of mUNO on St-PGA improved receptor targeting. Scale bars represent 20 µm.

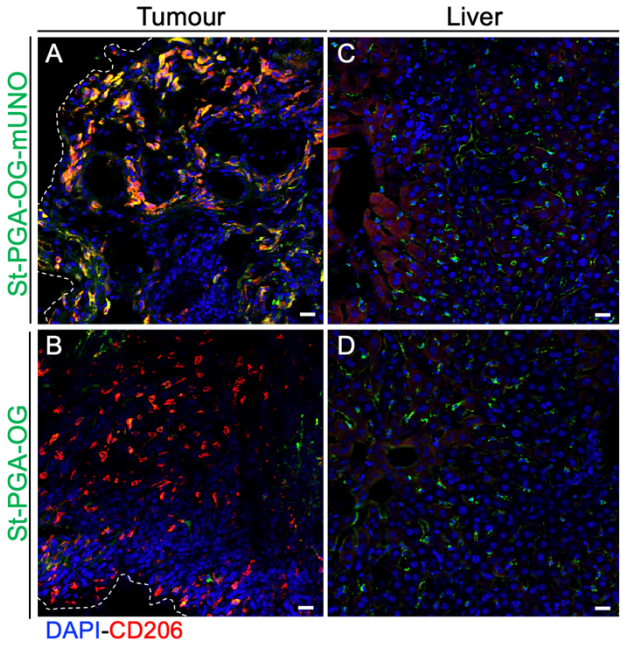


Fig. S6. St-PGA-OG-mUNO shows high homing to the M2 TAMs on the orthotopic TNBC model but higher accumulation in the liver, with a higher dose. St-PGA-OG-mUNO (0.82mg/0.5mL) or St-PGA-OG (0.7mg/0.5mL) was injected i.p. 10 days post tumour induction (s.c. injection of $1x10^6$ 4T1 cells), N=2. Nanoconjugates were circulated for 6h after which mice were sacrificed and organs collected for analysis. (**A**) St-PGA-OG-mUNO showed high colocalisation with CD206 whereas (**B**) St-PGA-OG showed minimal colocalisation. (**C, D**) Both nanoconjugates showed moderate accumulation in the liver. Scale bars represent 20 µm.

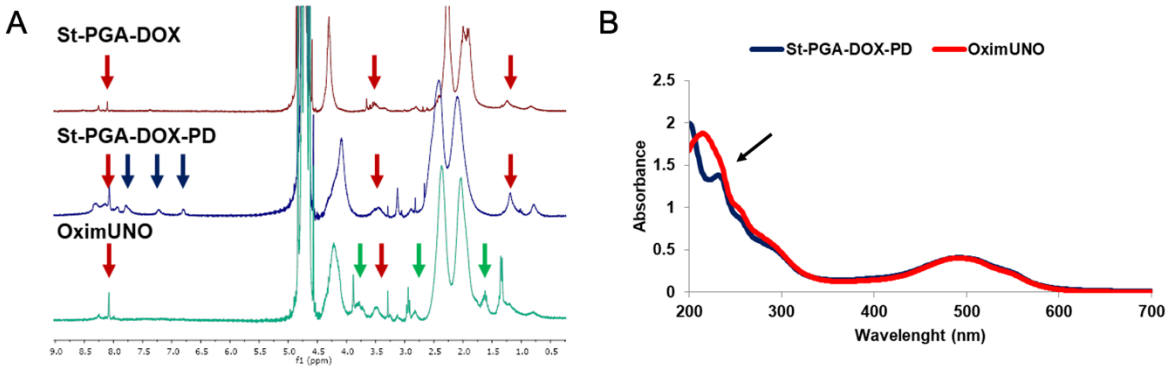
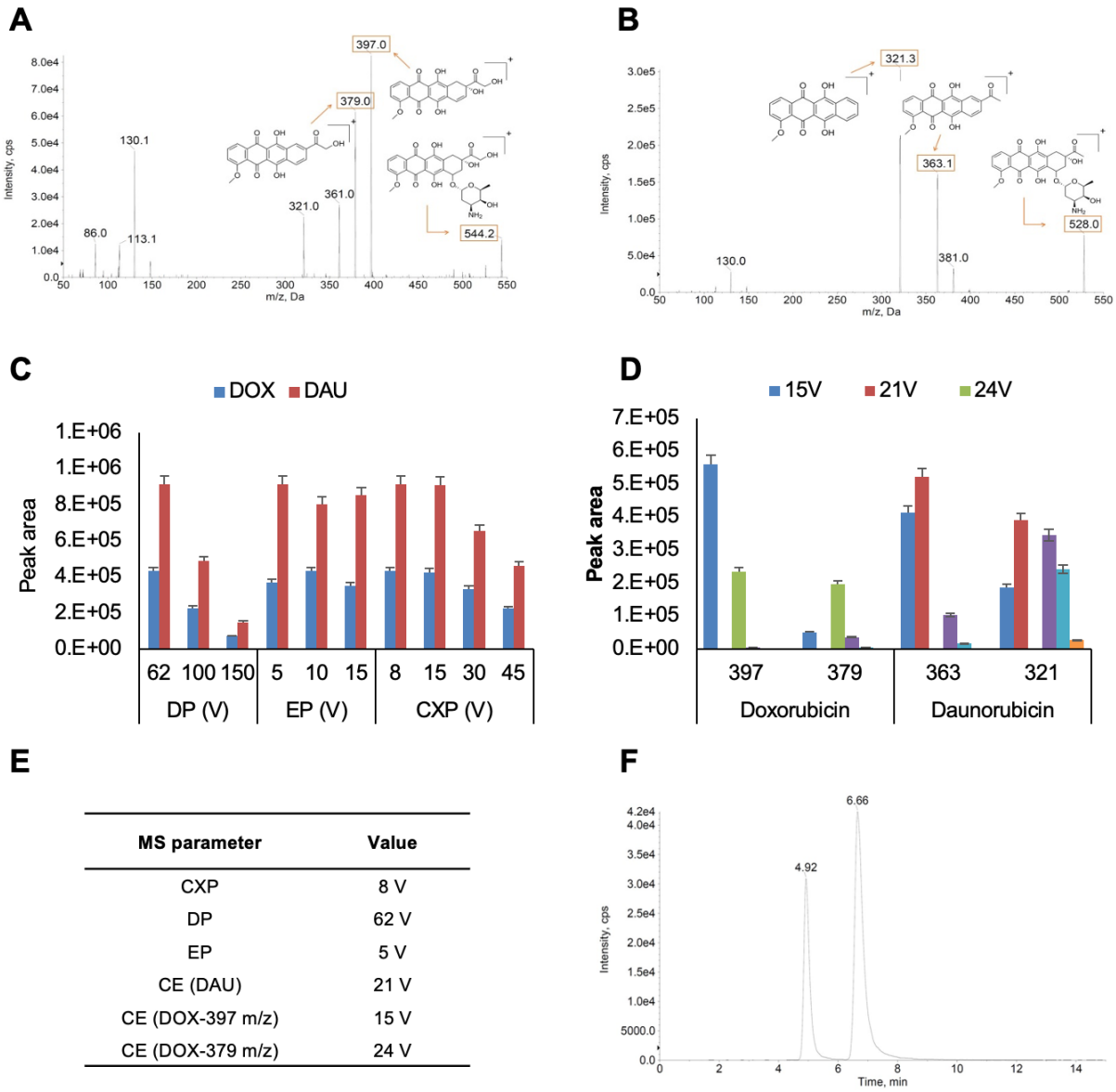


Fig. S7. Representative characterisation of St-PGA-DOX and OximUNO. (**A**) ^{1}H-NMR in D_2O of the nanoconjugates. Red arrows indicate the signals from DOX, blue arrows show the peaks from the pyridyl of the pyridyldithiol (PD), while green arrows indicate the signals from mUNO. (**B**) UV-Vis spectrum of St-PGA-PD-DOX (blue line) and OximUNO (red line) showing the displacement of pyridyl moiety at 260 nm by mUNO.



E

MS parameter	Value
CXP	8 V
DP	62 V
EP	5 V
CE (DAU)	21 V
CE (DOX-397 m/z)	15 V
CE (DOX-379 m/z)	24 V

G

	Recovery		Matrix effects		LOD (µg/ml)	LOQ (µg/ml)	Accuracy (%)
	DOX	DAU	DOX	DAU	DOX	DOX	DOX
dPBS 7.4	94.44%	68.25%	1.03	0.95			
acetate buffer pH 5	128.50%	67.66%	1.41	0.96	4.62	10.72	78-120
i.p. fluid	N/A	N/A	1.17	1.76			

Fig. S8. LC-MS method development for determination of DOX in drug release studies and stability studies of OximUNO in i.p. fluid and dPBS (**A**) MS-MS fragmentation spectra of DOX. (**B**) MS-MS fragmentation spectra of DAU. (**C**) Optimisation of MS parameter (average ± Sd, N=3). (**D**) Optimisation of CE for each mass transition of DOX and DAU (average ± Sd, N=3). (**E**) Final MS parameters. (**F**) Representative LC-MS chromatogram of DOX and DAU with t_r=4.92 min and t_r=6.66 min, respectively. (**G**) Method validation parameters. Error bars represent SE. Abbreviations: i.p. fluid – intraperitoneal fluid; DAU – daunorubicin; CXP –

collision exit potential; DP – declustering potential; EP – entrance potential; CE – collision energy; LOD – limit of detection; LOQ – limit of quantification; N/A – not applicable.

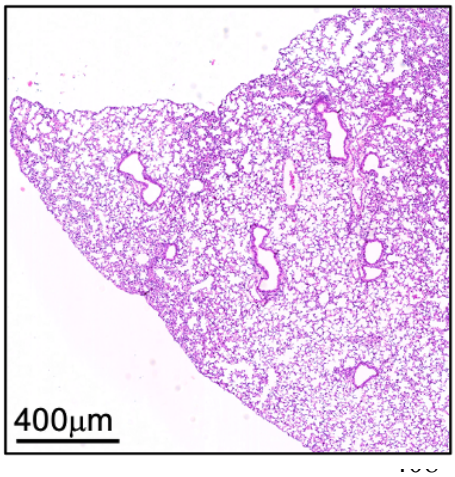


Fig. S9. H&E on healthy Balb/c mouse lung. Representative microscopy image from healthy female Balb/c mouse lung used as a control in H&E analysis showing the typical lung structure. Scale bar represents 400 µm.

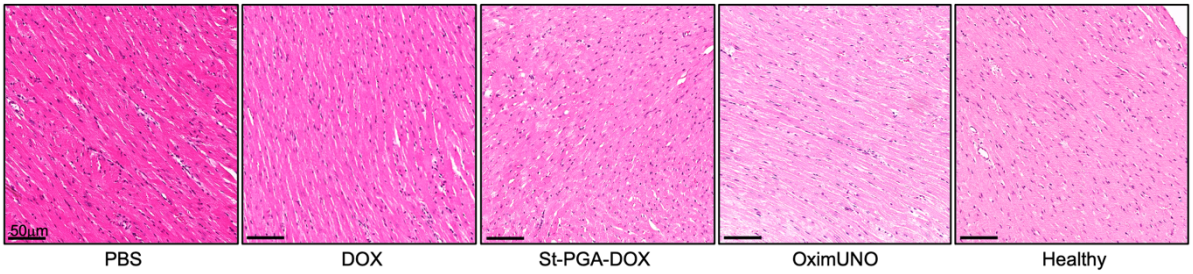


Fig. S10. H&E on hearts from monotherapy with orthotopic TNBC model. Hearts from all treatment groups were analysed with H&E for potential cardiotoxicity. All treatment groups showed no cardiotoxicity when analysed with H&E. The structure of heart tissue is similar to the structure seen with a healthy heart (farthest right). Scale bar represents 50 µm.

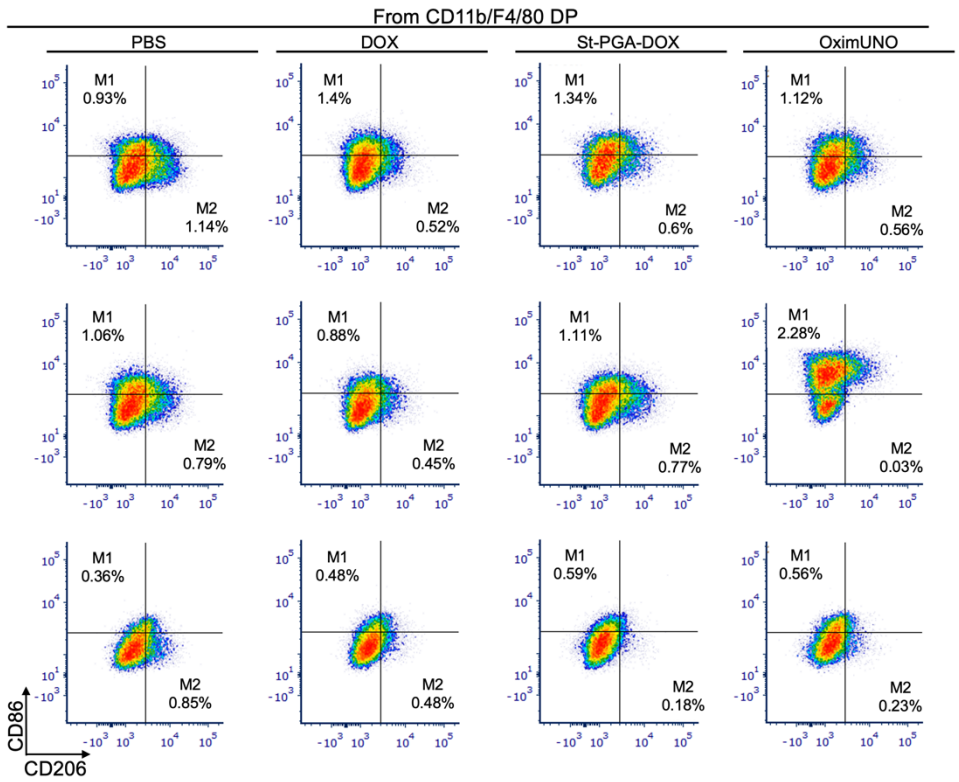


Fig. S11. Flow cytometry plots for M2 and M1 macrophages. Cytometry plots for the M2 and M1 macrophage populations of the treatment study shown in Figure 6. Percentages shown are from total cells. DP – double positive.

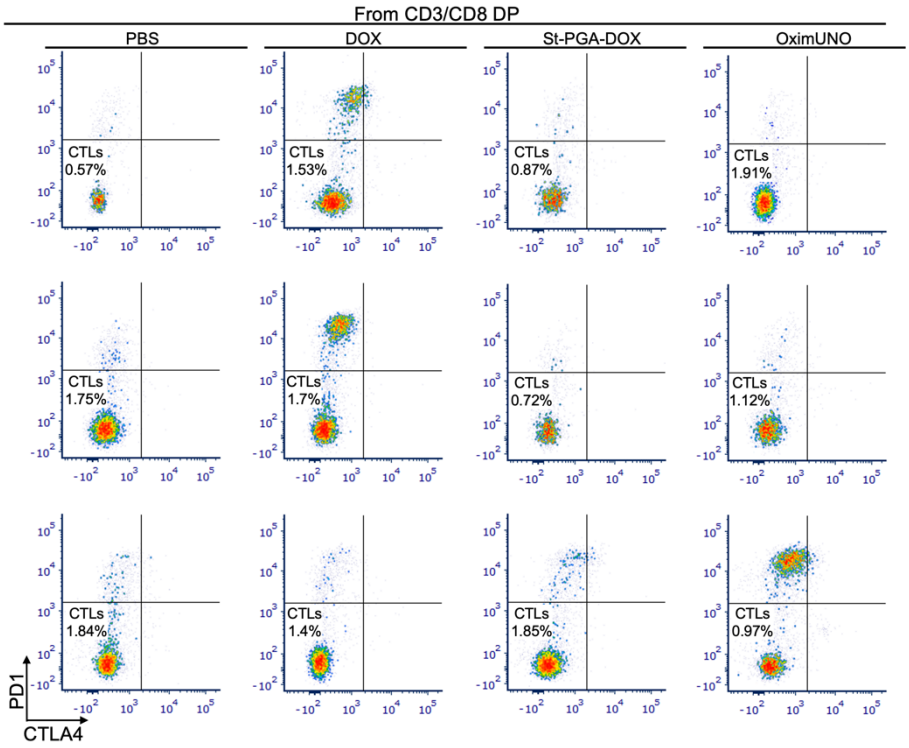
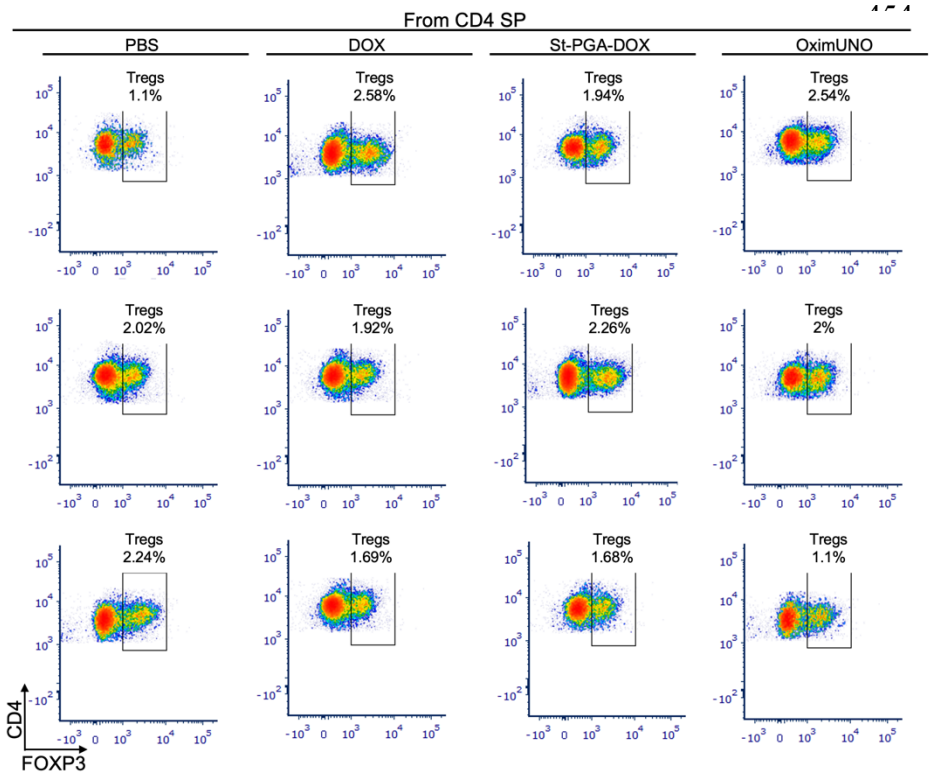
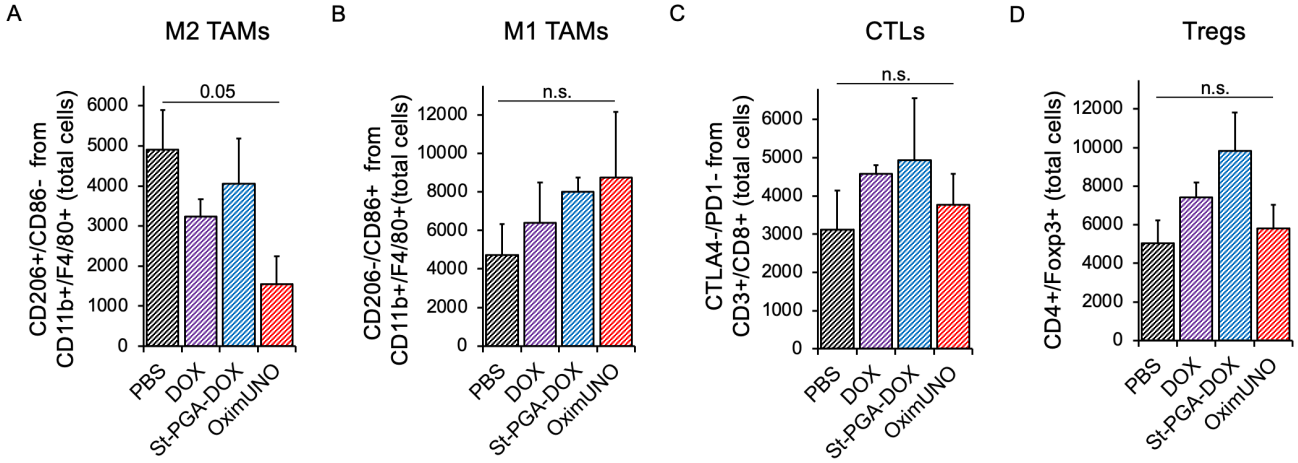


Fig. S12. Flow cytometry gating for cytotoxic T lymphocytes (CTLs). Cytometry plots for the CTLs populations of the treatment study shown in Figure 6. Percentages shown are from total cells. DP – double positive



471 **Fig. S13. Flow cytometry gating for T regulatory cells (Tregs).** Cytometry plots for the Treg populations of
472 the treatment study shown in Figure 6. Percentages shown are from total cells. SP – single positive
473



474
475 **Fig. S14. Flow cytometry analysis showing total cells.** Flow cytometry analysis showing total cells for (**A**) M2
476 TAMs, (**B**) M1 TAMs, (**C**) CTLs, and (**D**) Tregs. Error bars represent SE.
477
478
479
480
481
482

REFERENCES

1. Barz, M., Duro-Castano, A. & Vicent, M. J. A versatile post-polymerization modification method for polyglutamic acid: synthesis of orthogonal reactive polyglutamates and their use in “click chemistry”. *Polym. Chem.* **4**, 2989–2994 (2013).
2. Van Lysebetten, D. *et al.* Lipid-Polyglutamate Nanoparticle Vaccine Platform. *ACS Appl. Mater. Interfaces* **13**, 6011–6022 (2021).
3. Arroyo-Crespo, J. J. *et al.* Tumor microenvironment-targeted poly-L-glutamic acid-based combination conjugate for enhanced triple negative breast cancer treatment. *Biomaterials* **186**, 8–21 (2018).