Supplementary Information for

Tumor Immune Cell Targeting Chimeras (TICTACs) For Targeted Depletion of Macrophage-Associated Checkpoint Receptors

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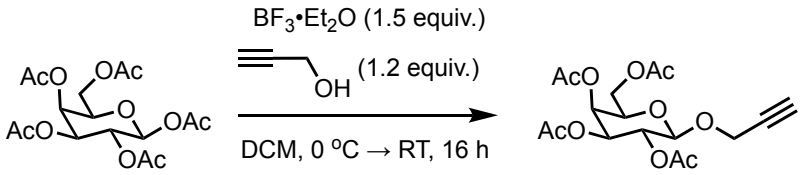
Chemical Analysis Instrumentation

Proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) spectra were taken with Neo-500 Bruker spectrometer operating at 500 MHz at 25 °C. Chemical shifts are reported in parts per million (ppm) with reference to the appropriate residual solvent signal. ^{1}H NMR: $CDCl_3$ (δ: 7.26 ppm), DMSO-d_6 (δ: 2.50 ppm), MeOD (δ: 3.31 ppm), D_2O (δ: 4.79 ppm). ^{1}H NMR multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), sept (septet), m (multiplet). Liquid chromatography mass spectrometry (LC-MS) analysis was performed on an Agilent 1260 Infinity II using acetonitrile (HPLC grade, Fisher, Waltham, MA) with 1% formic acid and Milli-Q Gradient ultrapure water (Millipore, Billerica, MA) with 1% formic acid. Preparative high performance liquid chromatography mass spectrometry (Prep-HPLC-MS) was performed on an Agilent 1290 Infinity II using acetonitrile with 1% formic acid and Milli-Q Gradient ultrapure water with 1% formic acid.

Synthesis of sodium propargyl 3-sulfonato-β-D-galactopyranoside

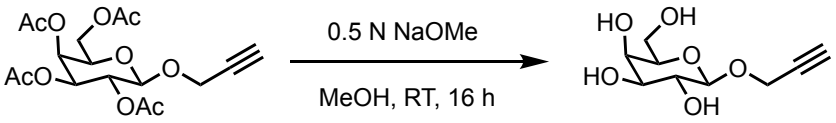
Propargyl 2,3,4,6-tetra-O-acetyl β-D-galactopyranoside (S1)[1]



S1

β-D-galactose pentaacetate (2 g, 5.1 mmol, 1 equiv.) and propargyl alcohol (0.36 mL, 6.12 mmol, 1.2 equiv.) were combined in a flame-dried 50 mL Schlenk flask in 20 mL of anhydrous DCM. The mixture was cooled to 0 °C, and $BF_3{\cdot}Et_2O$ (0.94 mL, 7.65 mmol, 1.5 equiv.) was added dropwise. The reaction was gradually warmed to RT and stirred overnight. The reaction was quenched with aq. $NaHCO_3$ (5 mL), stirred 20 min, diluted with water, and extracted with DCM (20 mL x 2). The combined organic phases were dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The product was purified by column chromatography (30% EtOAc/hexanes) to yield **S1** as a clear oil. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.40 (dd, *J* = 3.5, 1.1 Hz, 1H), 5.22 (dd, *J* = 10.4, 7.9 Hz, 1H), 5.06 (dd, *J* = 10.4, 3.4 Hz, 1H), 4.74 (d, *J* = 8.0 Hz, 1H), 4.38 (d, *J* = 2.3 Hz, 2H), 4.22 – 4.09 (m, 2H), 3.94 (td, *J* = 6.7, 1.2 Hz, 1H), 2.46 (t, *J* = 2.4 Hz, 1H), 2.15 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H).

Propargyl β-D-galactopyranoside (S2)[1]

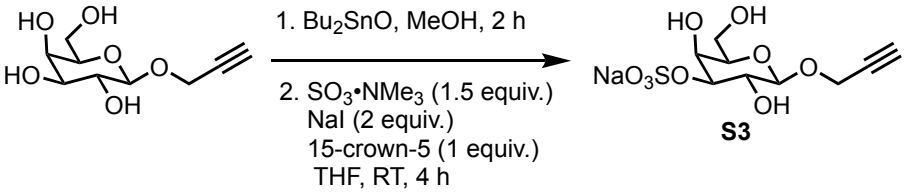


S2

To a solution of compound **S1** in anhydrous MeOH (10 mL) was added 0.5 N NaOMe solution (20 mL). The reaction was stirred at RT overnight and quenched with Amberlite IR-120 resin (H^+ form), filtered, then concentrated to obtain the crude product, which was a semi-solid/viscous oil. The crude product was triturated in pentane/Et_2O, filtered, and dried under high vacuum for 1 h to yield

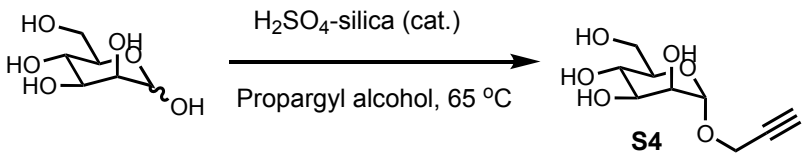
S2 as a crystalline white solid (R_f = 0.25 in 3:1 $CHCl_3$: MeOH). ^{1}H NMR (500 MHz, MeOD/Tol 1:1) δ 4.48 (d, *J* = 7.7 Hz, 1H), 4.44 (dd, *J* = 4.0, 2.5 Hz, 2H), 3.93 – 3.87 (m, 2H), 3.84 (dd, *J* = 11.4, 5.4 Hz, 1H), 3.72 (dd, *J* = 9.6, 7.8 Hz, 1H), 3.56 (dd, *J* = 9.7, 3.4 Hz, 1H), 3.50 (t, *J* = 6.1 Hz, 1H), 2.67 (t, *J* = 2.5 Hz, 1H).

Sodium propargyl 3-sulfonato-β-D-galactopyranoside (S3)[1]



Compound **S2** (324 mg, 1.48 mmol, 1 equiv.) and dibutyltin oxide (555 mg, 2.23 mmol, 1.5 equiv.) were dissolved in anhydrous MeOH (30 mL) and the reaction was heated to reflux for 2 h. The solvent was removed under reduced pressure, and the residue was taken up in anhydrous THF (20 mL). Sulfur trioxide trimethylamine complex (355 mg, 2.23 mmol, 1.5 equiv.) was first added, then NaI (445 mg, 2.97 mmol, 2 equiv.), then 15-crown-5 (0.3 mL, 1.48 mmol, 1 equiv.), and the reaction was stirred at RT for 4 h. The reaction was monitored with TLC (3:1 $CHCl_3$: MeOH). When the reaction was complete, $NaHCO_3$ (5 mL) and 5% Na2S2O3 (5 mL) were added. The mixture was concentrated *in vacuo*, then directly adsorbed onto silica in MeOH. The crude mixture was purified by column chromatography ($CHCl_3$, then $CHCl_3$: MeOH 9:1, $CHCl_3$: MeOH 5:1, then 1:1). Earlier impurities elute with 9:1 and 5:1. **S3** was isolated as a white foam (404 mg, 85% yield). ^{1}H NMR (500 MHz, MeOD/Tol 1:1) δ 4.50 (d, *J* = 7.7 Hz, 1H), 4.38 – 4.30 (m, 4H), 3.87 – 3.72 (m, 3H), 3.49 (t, *J* = 6.0 Hz, 1H), 2.61 (q, *J* = 1.8 Hz, 1H).

Synthesis of Propargyl α-D-mannose (S4)[2]

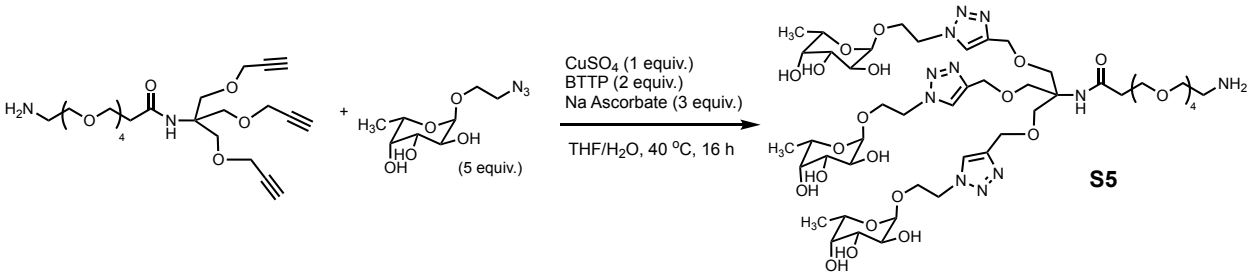


To mannose (1 g, 1 equiv.) in neat propargyl alcohol (1.6 mL) was added H_2SO_4-silica* (27.8 mg, cat.). The reaction was heated to 65 °C, during the course of which the mixture became homogeneous. The crude mixture was first eluted through a short silica column with DCM to remove excess propargyl alcohol, then purified by column chromatography (10% MeOH in DCM) to isolate the α-anomer **S4** as a white solid. ^{1}H NMR (500 MHz, MeOD) δ 4.96 (d, *J* = 1.7 Hz, 1H), 4.27 (d, *J* = 2.4 Hz, 2H), 3.86 – 3.78 (m, 2H), 3.73 – 3.59 (m, 3H), 3.51 (ddd, *J* = 10.8, 5.1, 2.1 Hz, 1H), 2.85 (t, *J* = 2.4 Hz, 1H).

**H_2SO_4-slica was prepared as the following:*

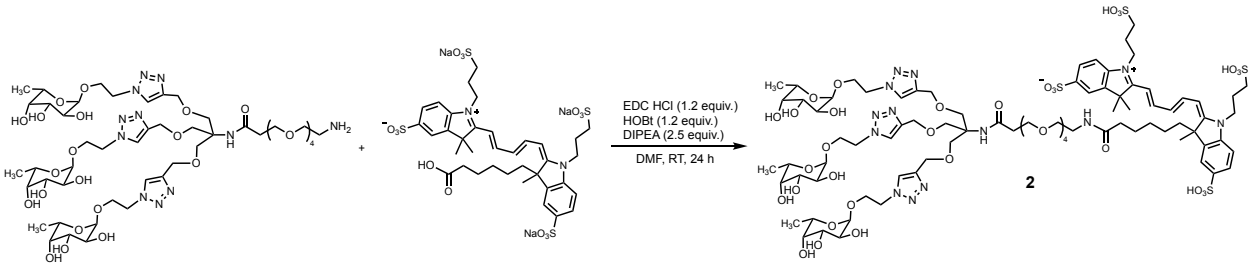
10 g silica was added to diethyl ether (50 mL) and mixed to form a slurry. To this mixture was added concentrated H2SO4 (3 mL), and the mixture was shaken/stirred for 5 min. The solvent was removed under reduced pressure until silica was free-flowing, then dried in the oven for at least 3 h.

Amino-PEG4-tris-α-L-fucose (S5)



To a 1-dram vial equipped with a magnetic stir bar was added amino-PEG_4-tris-alkyne (10 mg, 0.02 mmol, 1 equiv.) and 2-azidoethyl α-L-fucopyranoside (23.3 mg, 0.14 mmol, 5 equiv.) as a solution in 0.5 mL of degassed 1:1 THF/ddH_2O. In a separate one-dram vial, $CuSO_4$ (5 mg, 0.02 mmol, 1 equiv.), BTTP (17.22 mg, 0.04 mmol, 2 equiv.), and sodium ascorbate (11.9 mg, 0.06 mmol, 3 equiv.) were combined in 0.2 mL of ddH_2O, then added to the reaction mixture. The solution was heated to 40 °C and stirred for 16 h. The mixture was purified by preparative HPLC using a 0-30% acetonitrile gradient over 11 min, and the product was isolated as a white solid after lyophilization (15.5 mg, 65% yield). ^{1}H NMR (500 MHz, MeOD) δ 8.11 (s, 3H), 4.76 (d, *J* = 3.8 Hz, 3H), 4.71 – 4.60 (m, 5H), 4.58 (s, 8H), 4.06 (ddd, *J* = 11.2, 7.9, 3.6 Hz, 3H), 3.83 (ddd, *J* = 10.9, 5.6, 3.6 Hz, 3H), 3.78 – 3.61 (m, 22H), 3.59 (s, 4H), 3.58 – 3.55 (m, 3H), 3.41 (q, *J* = 6.8 Hz, 4H), 3.14 (t, *J* = 5.1 Hz, 2H), 2.48 (t, *J* = 6.0 Hz, 2H), 1.10 (d, *J* = 6.6 Hz, 9H). LC-MS *m/z*: 1182.4 ($M+H^+$), 591.9 ($M+2H^+$).

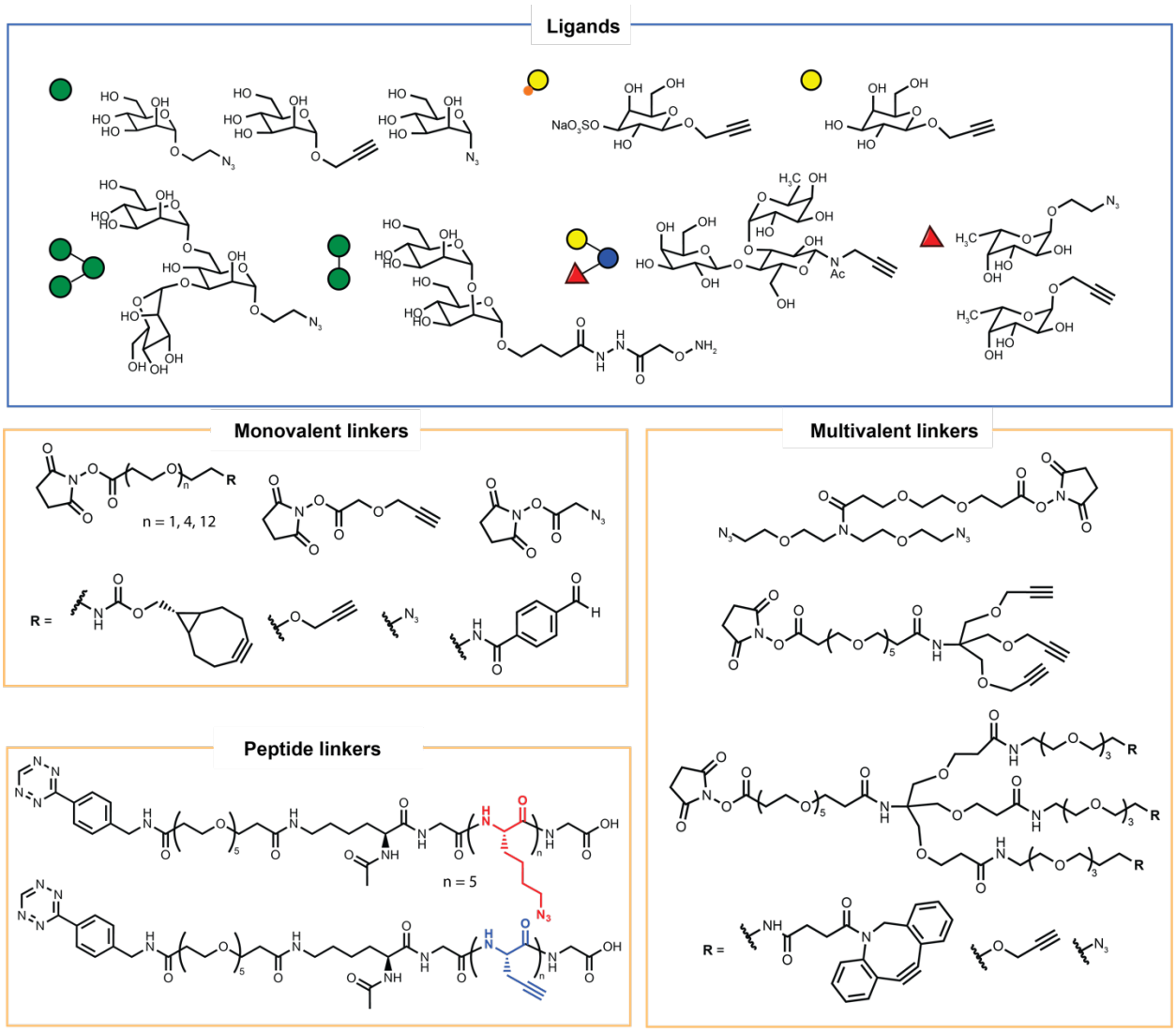
Tris-fucose-647 (**2**)



To a 1-dram vial equipped with a magnetic stir bar was added **S5** (3.62 mg, 0.0031 mmol, 1 equiv.), APDye Fluor 647 acid (3 mg, 0.0032 mmol, 1 equiv.), and 1-hydroxy-7-azabenzotriazole (0.52 mg, 0.0038 mmol, 1.2 equiv.) as a solution in 0.2 mL DMF. To this mixture was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.73 mg, 0.0038 mmol, 1.2 equiv.) and diisopropylethylamine (1.33 µL, 0.0077 mmol, 2.5 equiv.). The mixture was stirred for 24 h at room temperature. The crude mixture was purified by prep-HPLC using a 5-95% acetonitrile gradient over 11 min, and the product was isolated as a bright blue solid after lyophilization. LC-MS *m/z*: 1010.4 ($M-2H^+$), 673.2 ($M-3H^+$).

Antibodies and Usage Table

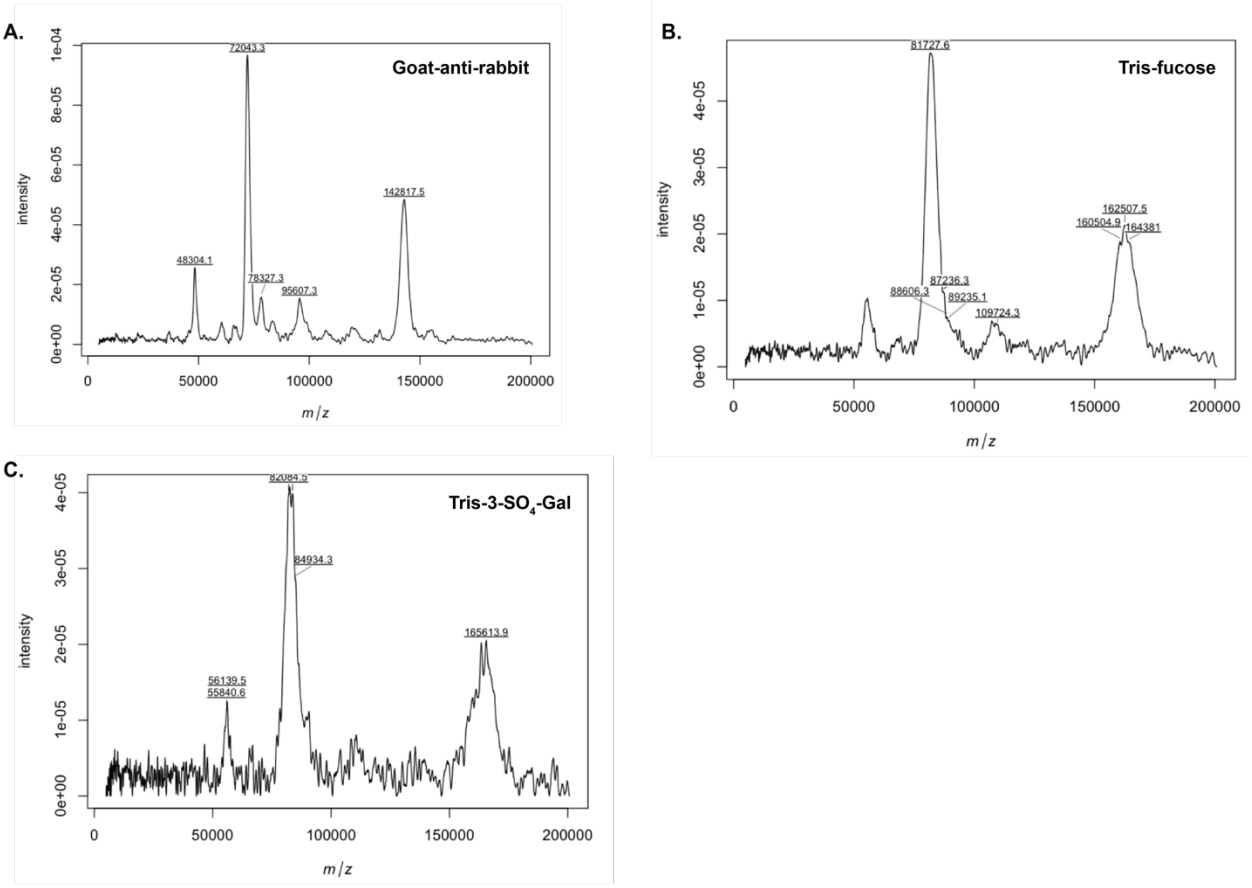
Antibody	Source (Cat #)	Usage and Dilution
Hamster anti-CD54	BD-Biosciences (550287)	Functional
Rat anti-CD54	BioXCell (BE0020-1)	Flow cytometry (3 µg/mL)
Rabbit anti-CD54	Proteintech (10020-1-AP)	WB, 1:1000
Rabbit anti-SIRPa	Proteintech (14482-1-AP)	WB, 1:1000
Recombinant Rabbit anti-SIRPa Clone #012	SinoBiological (50956-R012)	Functional, Flow cytometry
Recombinant Rabbit anti-SIRPa Clone #SF57	SinoBiological (custom order)	Functional
Recombinant Rabbit anti-SIRPa Clone #001	Thermo Fisher (MA5-29806)	Functional, Flow cytometry
Rat anti-SIRPa, Clone P84	BioXCell (BE0322)	Functional, Flow cytometry (3 µg/mL)
Recombinant Mouse CD47-Fc Chimera	Biolegend (785506)	Flow cytometry (30 µg/mL)
Rat anti-mouse IL-4	BioXCell (BE0199)	Functional
Goat anti-rabbit IgG	Jackson ImmunoResearch (111-005-144)	Functional
Polyclonal rabbit IgG	BioXCell (BE0095)	Functional
Rat anti-CD206 APC	Thermo Fisher (17-2061-82)	Flow cytometry (2 µg/mL)
Mouse anti-CD206 APC	Thermo Fisher (17-2069-42)	Flow cytometry (5 µL/test)
Rat anti-CD206 Alexa Fluor 488	Biolegend (141710)	Flow cytometry (5 µg/mL)
TruStain FcX (anti-mouse CD16/32)	Biolegend (101320)	Flow cytometry (2 µg/mL)
Human BD Fc Block	BD Biosciences (564219)	Flow cytometry (5 µg/mL)



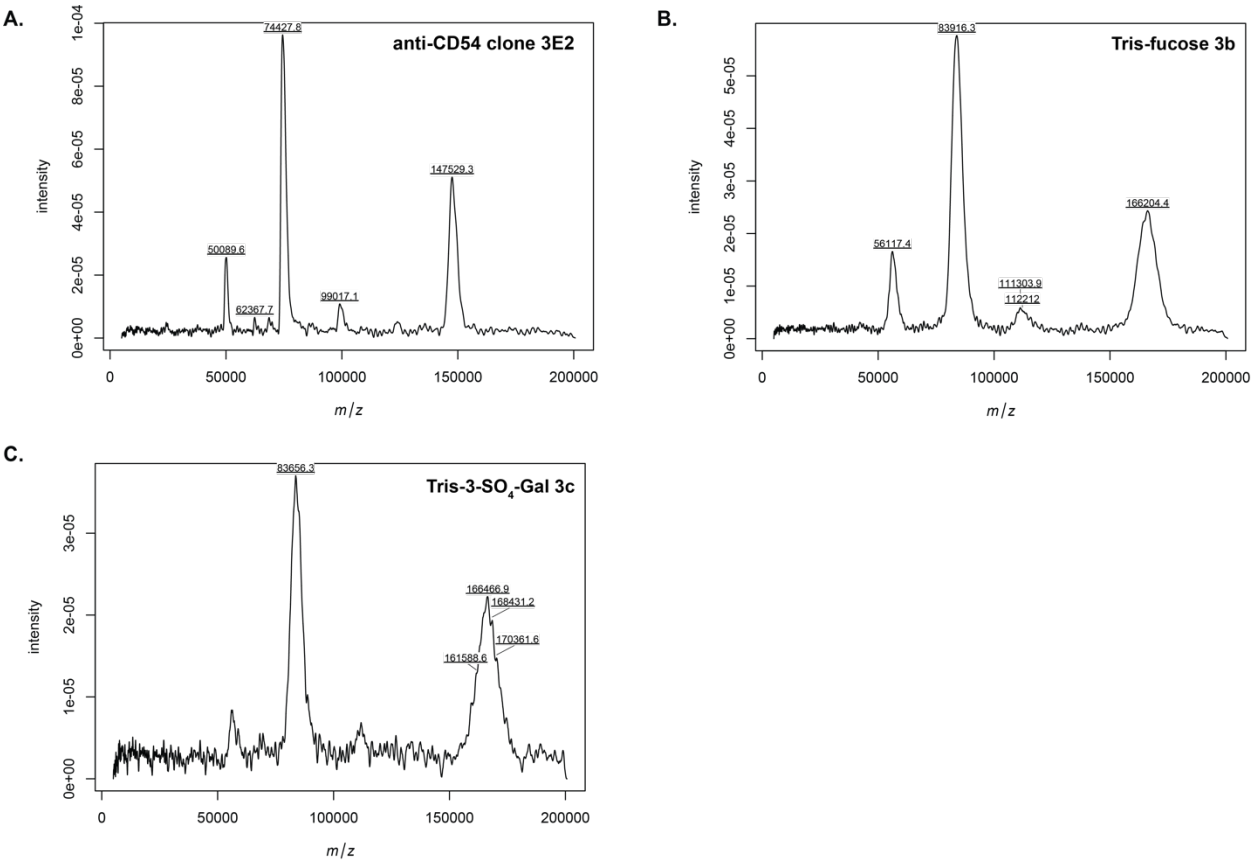
Supplementary Figure 1. Carbohydrate ligands and linkers used to generate TICTAC library. The antibody-carbohydrate conjugates were generated first by conjugating the linkers to the antibody, either through direct NHS coupling (monovalent and multivalent linkers) or via reaction with NHS-PEG_3-trans-cyclooctene (TCO) followed by an inverse Diels-Alder coupling for the peptide linkers. Carbohydrate ligands were added in excess and coupled to the linker through copper-catalyzed/copper-free click reaction or oxime ligation.

MALDI-MS Data for Antibody Conjugates

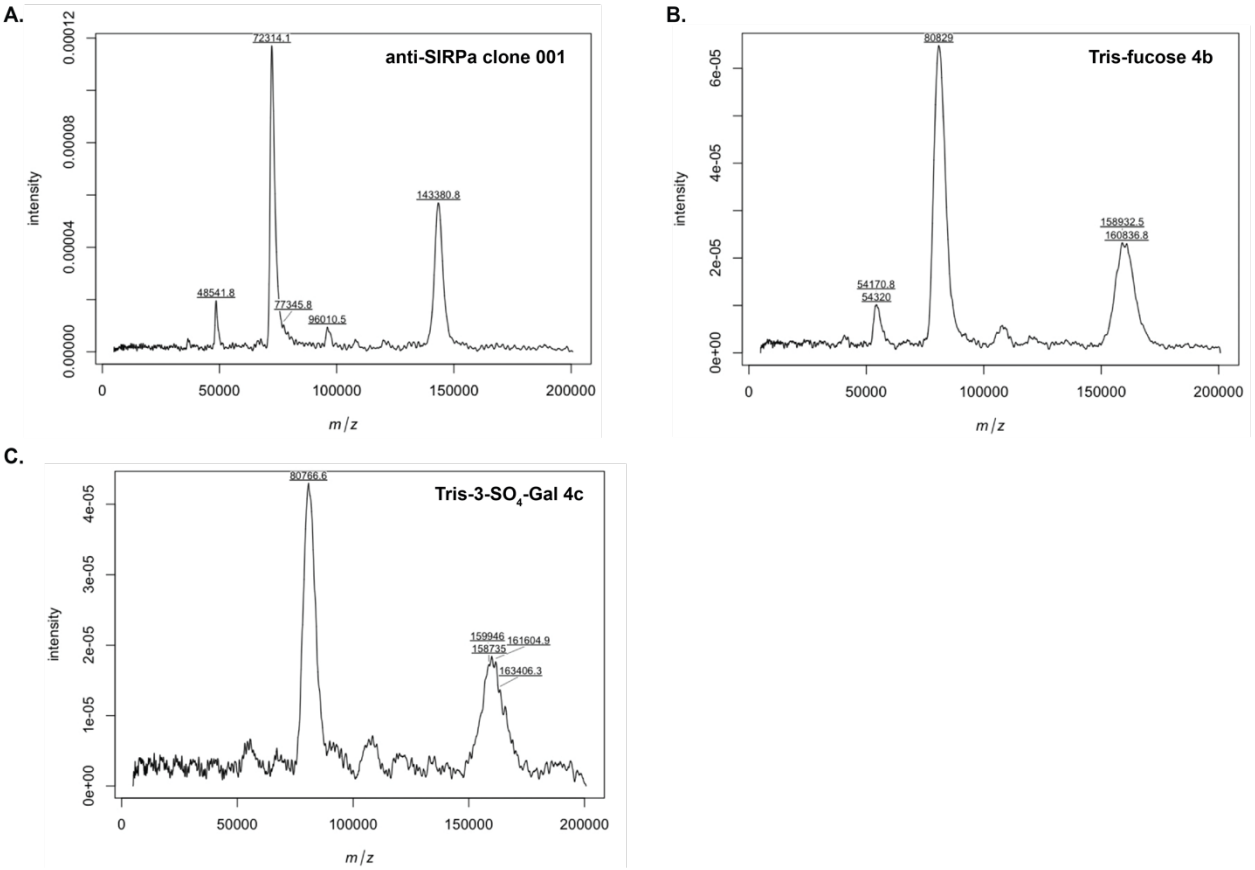
Data were processed using MALDI-Quant package.[3] The following spectra are average combined spectra of three or more measurements of two identically prepared samples.
The average ligand antibody ratio (LAR) was determined by subtracting the intact mass of the conjugated antibody from the unconjugated antibody and dividing by the molecular weight of the ligand: tris fucose = 1237.57, tris-3-SO_4-Gal = 2201.49.



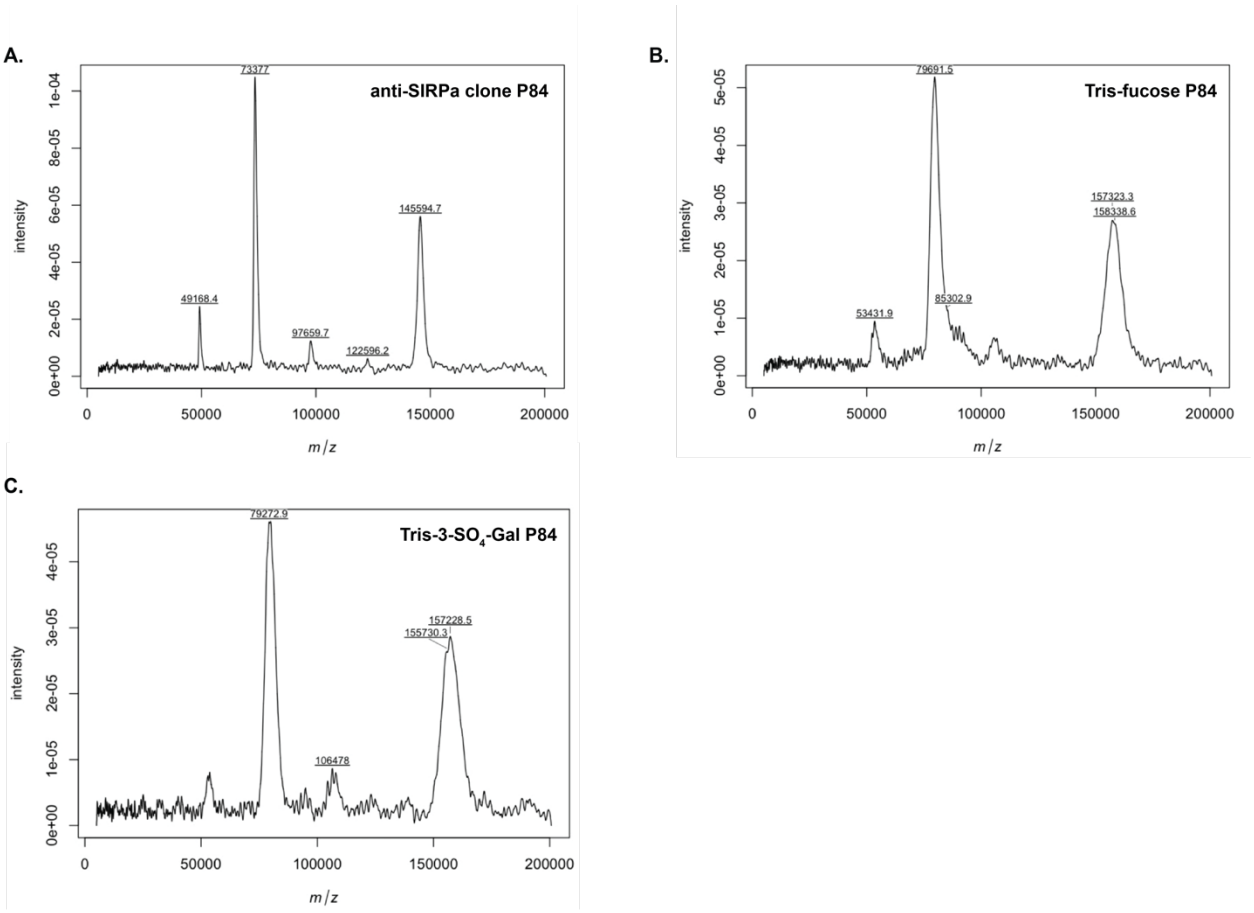
Supplementary Figure 2. MALDI-MS characterization of goat-anti-rabbit antibody conjugates. **A.** Combined spectrum of unconjugated goat-anti-rabbit antibody; **B.** Combined spectrum of tris-fucose conjugated goat-anti-rabbit antibody, where the average LAR is 16; **C.** Combined spectrum of tris-3-SO_4-Gal conjugated goat anti-rabbit antibody, where the average LAR is 10.



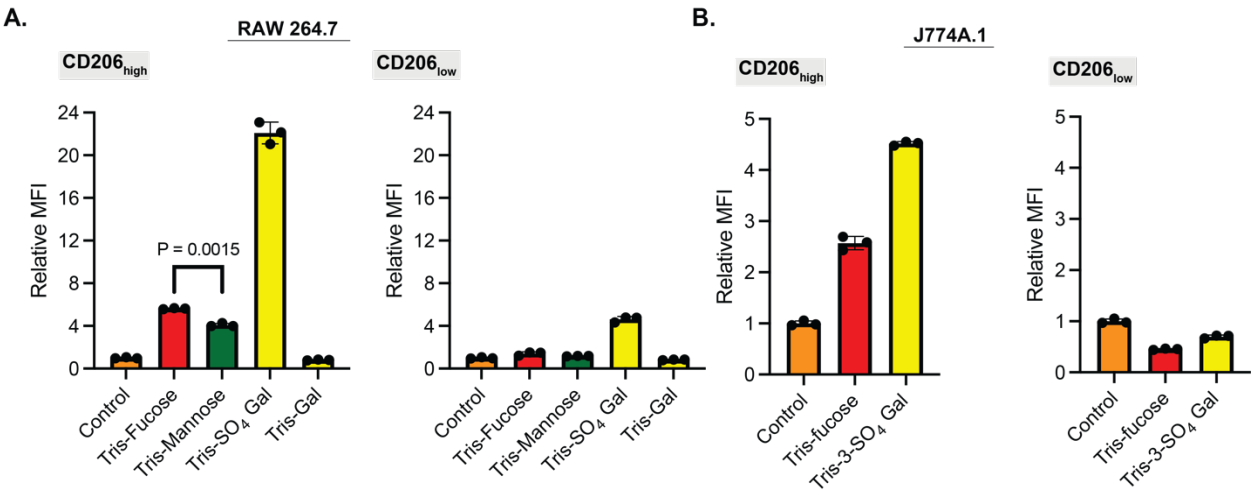
Supplementary Figure 3. MALDI-MS characterization of anti-CD54 antibody conjugates. **A.** Combined spectrum of unconjugated anti-CD54 antibody; **B.** Combined spectrum of tris-fucose conjugated anti-CD54 antibody **3b**, where the average LAR is 15; **C.** Combined spectrum of tris-3-SO_4-Gal anti-CD54 antibody **3c**, where the average LAR is 9.



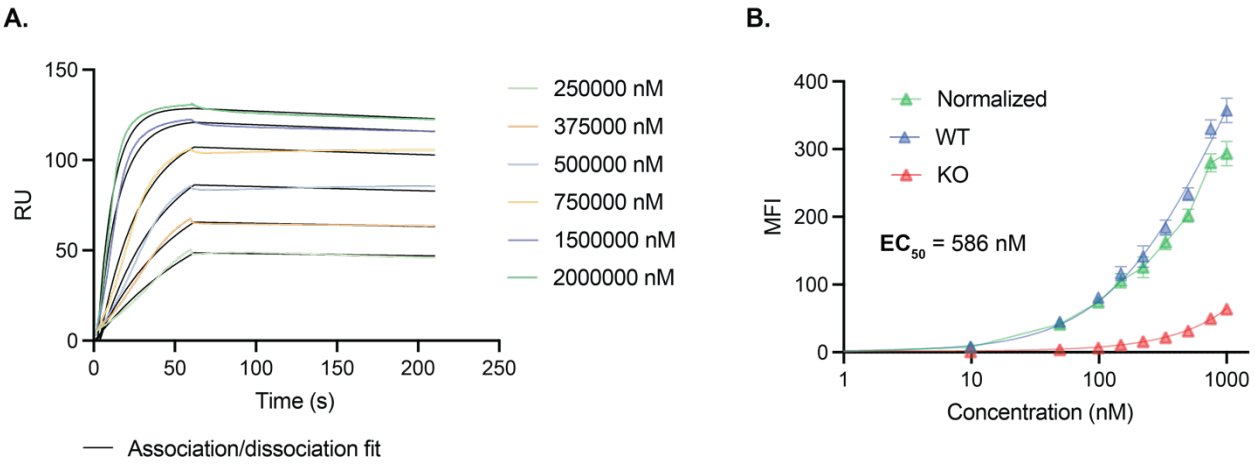
Supplementary Figure S4. MALDI-MS characterization of anti-SIRPα antibody conjugates. **A.** Combined spectrum of unconjugated anti-SIRPα antibody (clone 001); **B.** Combined spectrum of tris-fucose conjugated anti-SIRPα antibody **4b**, where the average LAR is 14; **C.** Combined spectrum of tris-3-SO_4-Gal anti-SIRPα antibody **4c**, where the average LAR is 8.



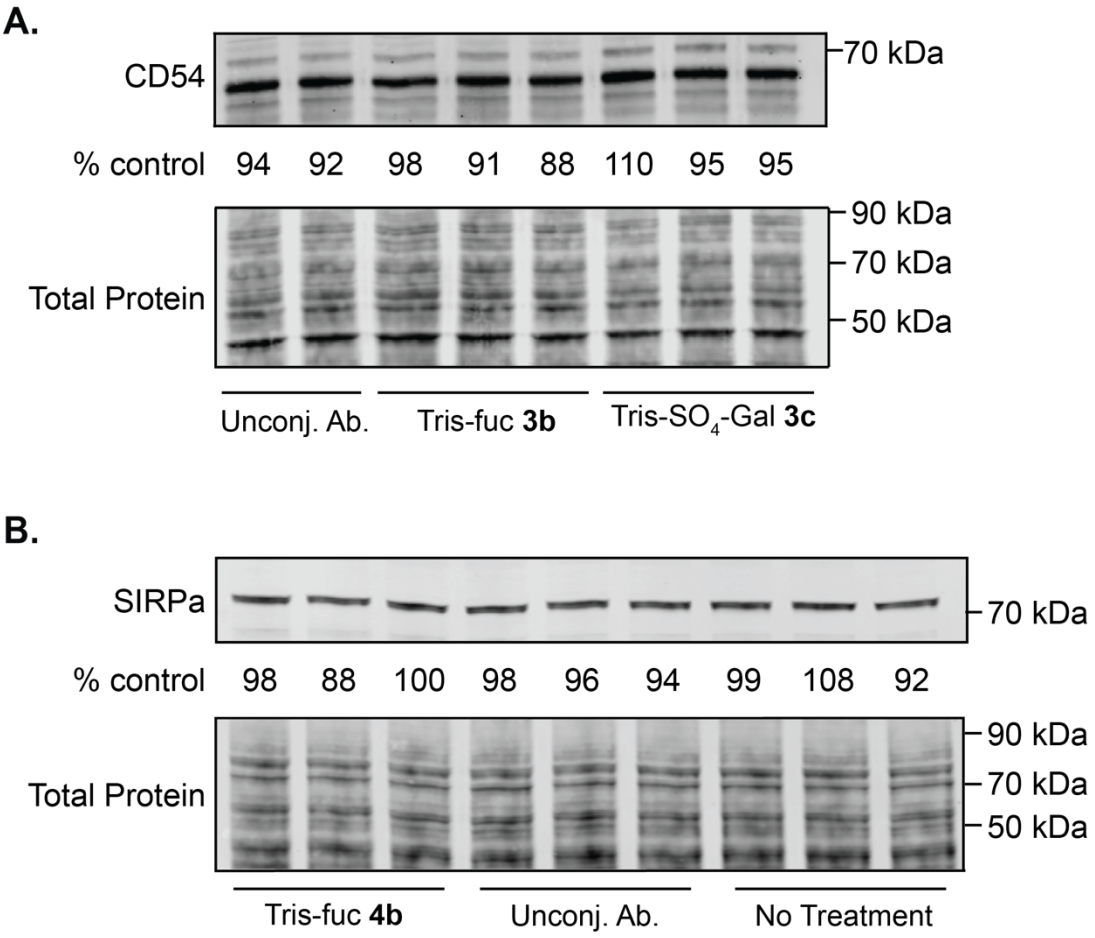
Supplementary Figure S5. MALDI-MS characterization of anti-SIRPα antibody conjugates. **A.** Combined spectrum of unconjugated anti-SIRPα antibody (clone P84); **B.** Combined spectrum of tris-fucose conjugated anti-SIRPα antibody **P84**, where the average LAR is 9; **C.** Combined spectrum of tris-3-SO_4-Gal anti-SIRPα antibody **P84**, where the average LAR is 5.



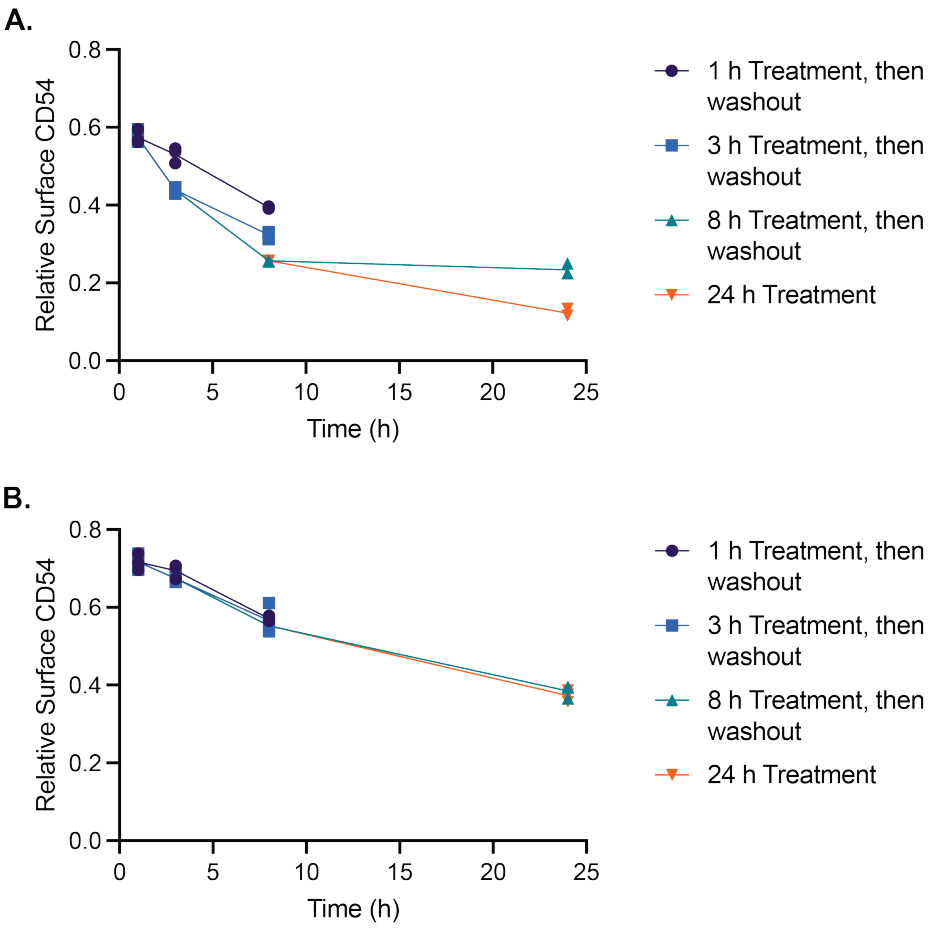
Supplementary Figure S6. Additional data for TICTAC-mediated internalization of fluorescent rabbit IgG into M2-polarized macrophages. **A.** Extended time periods (8 h) improves TICTAC-mediated uptake into RAW264.7 cells. **B.** Tris-fucose and Tris-3-SO_4-Gal TICTACs induce uptake of fluorescent IgG in M2-polarized J774A.1 cells. Error bars represent the SD from 3 independent experiments. For **A**, *P* values were determined by Welch's two-tailed *t*-tests.



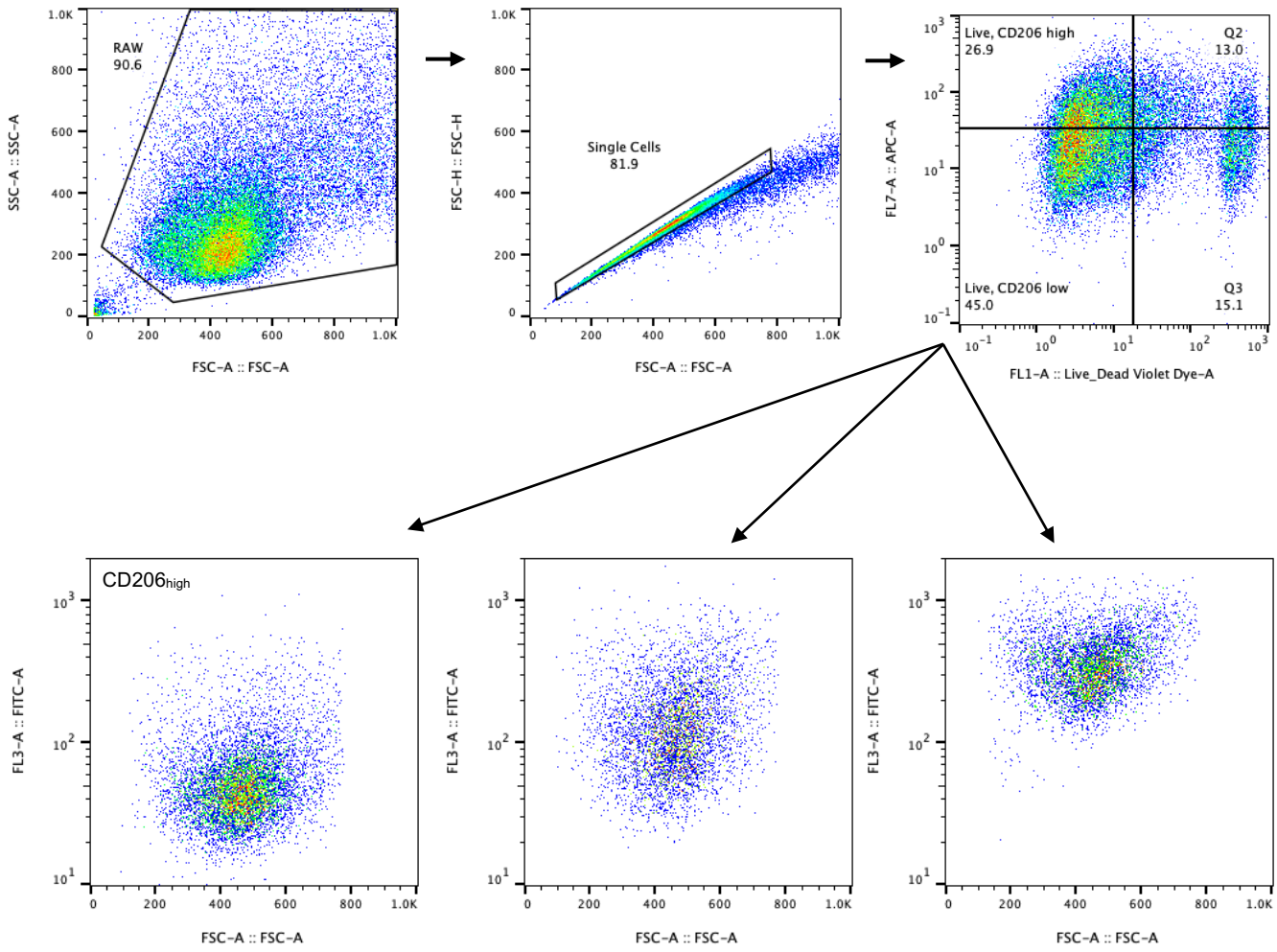
Supplementary Figure S7. Binding data for small molecule tris-fucose ligand. **A.** SPR binding curves and fitted association/dissociation curves for the interaction of **S1** with immobilized mouse CD206 protein. The K_d was calculated to be 4.58 μM, confirming binding; however, due to the extremely slow dissociation rate, the true K_d is likely to be lower. **B.** As an alternative readout for binding, EC_{50} of tris-fuc-647 (**2**) binding was determined. Wild-type (WT) and CD206 knockout (KO) RAW264.7 cells were incubated with variable concentrations of **2** for 2 h at 37 °C, then the mean fluorescence intensity (MFI) was determined by flow cytometry. The normalized curve was generated by subtracting the WT MFIs from KO (non-specific binding) MFIs. The EC_{50} was determined to be 586 nM.



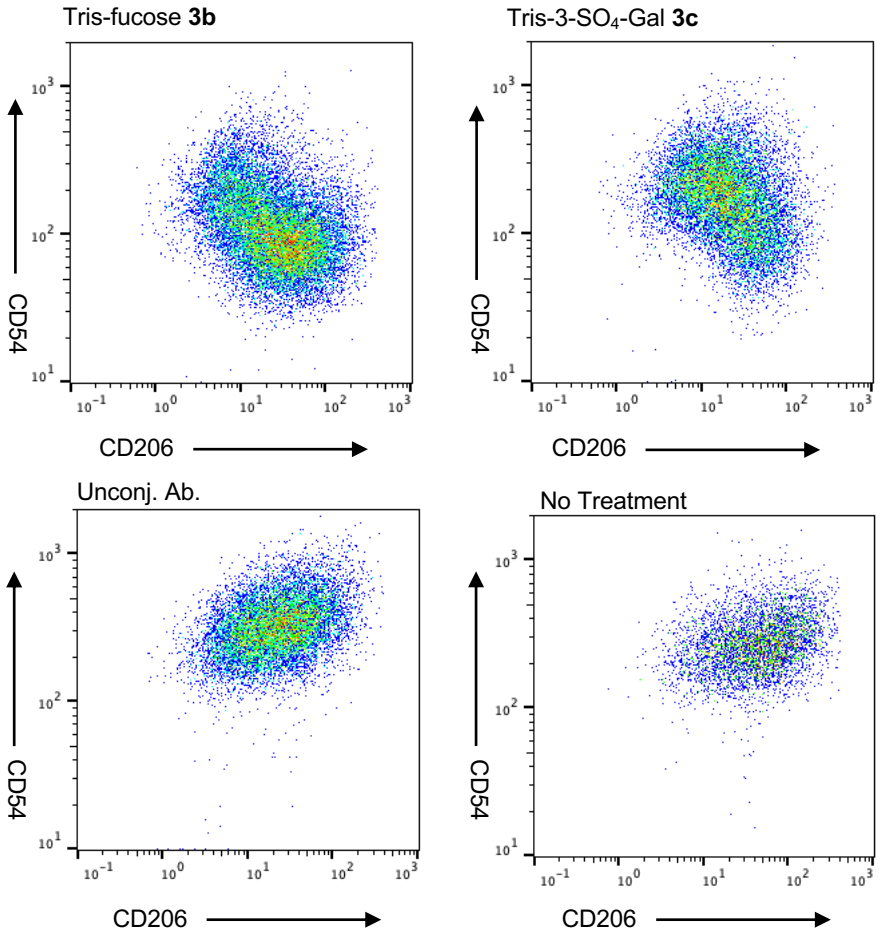
Supplementary Figure S8. Western blot quantification of total protein levels of CD54 and SIRPα after treatment with TICTACs or unconjugated antibody control. **A.** Treatment of RAW264.7 cells with 25 nM **3b** and **3c** over 24 h results in no significant change over treatment with the unconjugated antibody. **B.** Treatment of RAW264.7 cells with 25 nM **4b** over 24 h results in no significant change over treatment with the unconjugated antibody.



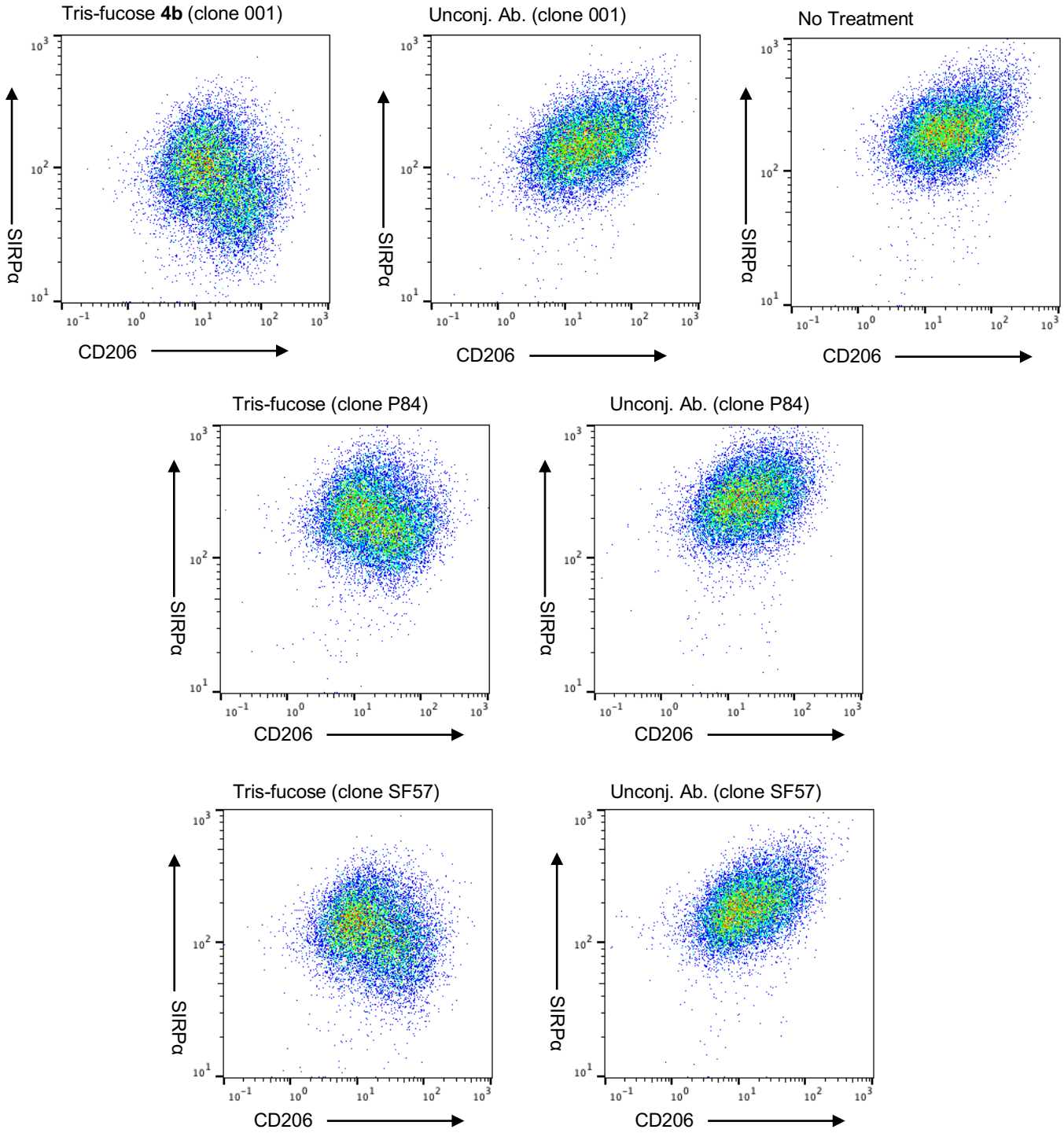
Supplementary Figure S9. Washout with M2-polarized RAW264.7 cells where cells were first exposed to media containing **3b**, **3c**, or the unconjugated antibody for 1 h, 3 h, or 8 h (treatment time), then allowed to grow in TICTAC or antibody-free media for varying time periods (washout period). **A.** Washout experiments for **3b**, where surface CD54 levels are normalized to the unconjugated antibody control. **B.** Washout experiments for **3c**, where surface CD54 levels are normalized to the unconjugated antibody control.



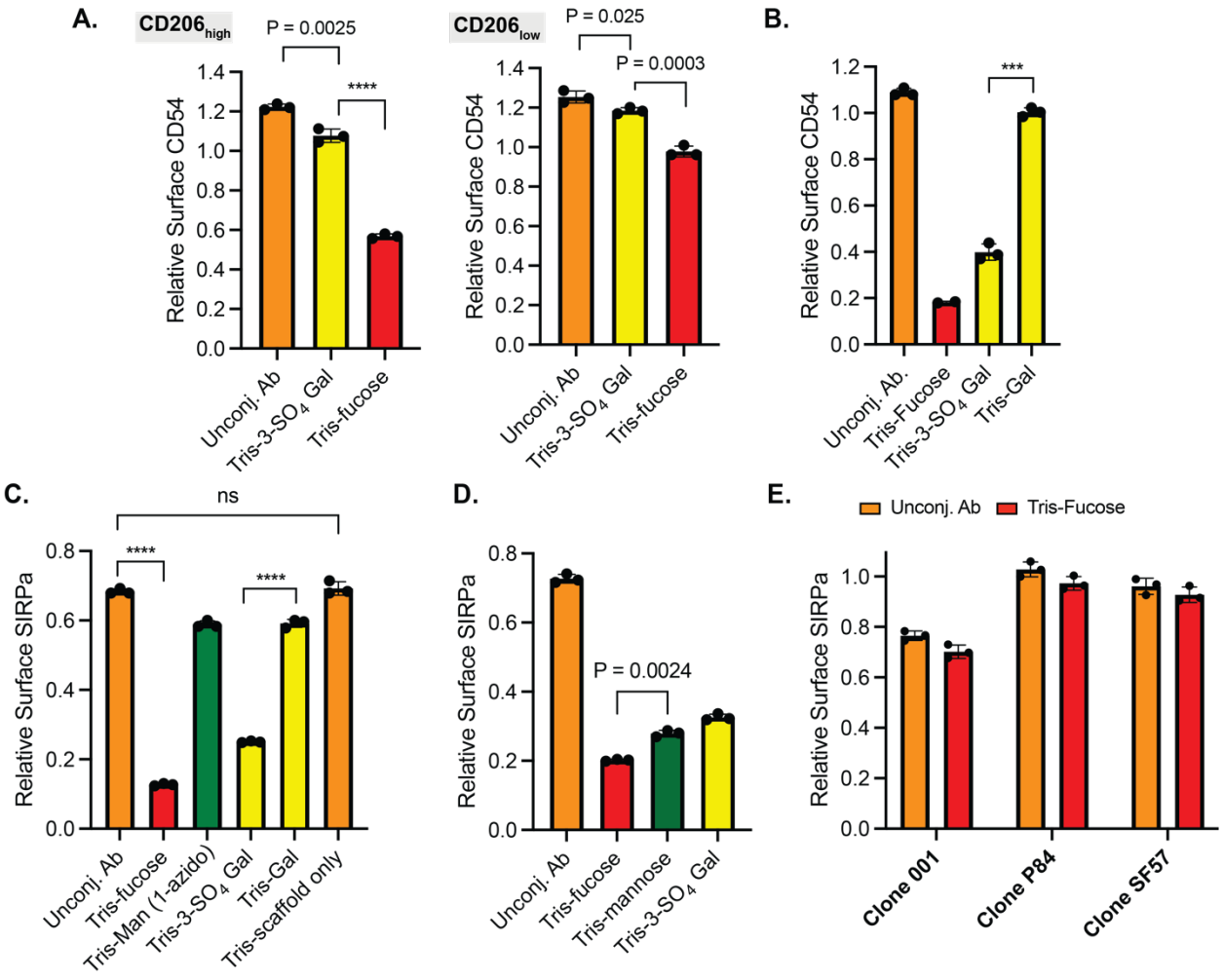
Supplementary Figure S10. Representative gating strategy for flow cytometry experiments. Data shown is for RAW264.7 cells treated with anti-CD54 antibody or TICTACs, gated for $CD206_{high}$ cells. CD206 levels correspond to APC, and CD54 levels correspond to FITC.



Supplementary Figure S11. Correlation between CD206 expression levels and CD54 after treatment of M2-polarized RAW264.7 cells with 25 nM anti-CD54 **3b**, **3c**, or unconjugated antibody for 24 h.



Supplementary Figure S12. Correlation between CD206 expression levels and SIRPα after treatment of M2-polarized RAW264.7 cells with 25 nM anti-SIRPα TICTACs or unconjugated antibody for 24 h.



Supplementary Figure S13. Additional control experiments for TICTAC-mediated downmodulation of membrane targets. **A.** Downmodulation of cell-surface CD54 in M2-polarized J774A.1 cells determined by live cell flow cytometry following 24 h of treatment with 25 nM unconjugated anti-CD54 antibody or conjugates. **B.** 24 h treatment of 25 nM anti-CD54 tris-gal conjugate results in no surface CD54 downmodulation in RAW264.7 cells. **C.** 24 h treatment of 25 nM anti-SIRPα tris-gal conjugate, tris-alkyne linker only conjugate (tris-scaffold only), and tris-man (anomeric azido-mannose) conjugate do not downmodulate surface SIRPα in RAW264.7 cells. **D.** Anti-SIRPα tris-fucose **4b** outperforms the corresponding tris-mannose conjugate in downregulating surface SIRPα. **E.** CD206 knockout ablates the activity of anti-SIRPα TICTACs for clones 001, P84, and SF57. Error bars represent the SD from 3 independent experiments. For **A**-**D**, *P* values were determined by Welch's two-tailed *t*-tests. Statistical significance was defined as $P<0.05$, and the asterisks *** indicates $P<0.001$, and **** indicates $P<0.0001$.

References:

(1) Zhao, J.; Liu, Y.; Park, H.-J.; Boggs, J. M.; Basu, A. Carbohydrate-Coated Fluorescent Silica Nanoparticles as Probes for the Galactose/3-Sulfogalactose Carbohydrate–Carbohydrate Interaction Using Model Systems and Cellular Binding Studies. *Bioconjug. Chem.* **2012**, *23* (6), 1166–1173.

(2) Krabicová, I.; Dolenský, B.; Řezanka, M. Selectivity of 1-O-Propargyl-d-Mannose Preparations. *Molecules* **2022**, *27* (5), 1483.

(3) Gibb, S.; Strimmer, K. MALDIquant: A Versatile R Package for the Analysis of Mass Spectrometry Data. *Bioinformatics* **2012**, *28* (17), 2270–2271.