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Supplementary information

Augmented lipid-nanoparticle-mediated in vivo genome editing in the lungs and spleen by disrupting Cas9 activity in the liver

In the format provided by the authors and unedited

a

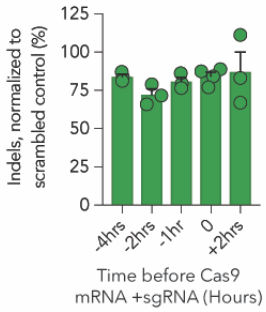
iOligo-A: GCTATTTCTAGCTCTAAAAC
iOligo-B: GACTAGCCTTATTTTAACTT
iOligo-C: ACTTTTTCAAGTTGATAACG
iOligo-D: AAAGCACCGACTCGGTGCCA
Scramble: GTATGACTTACGACTTATAT

b

sgICAM2: 5'-asasgsACGGACAGGCACCUACGGUUUUAGAgc
uagaaauagcAAGUUAAAAUAAGGCUAGUCCGUUA
UCAacuugaaaaaguggcaccgagucggugcusususu-3'

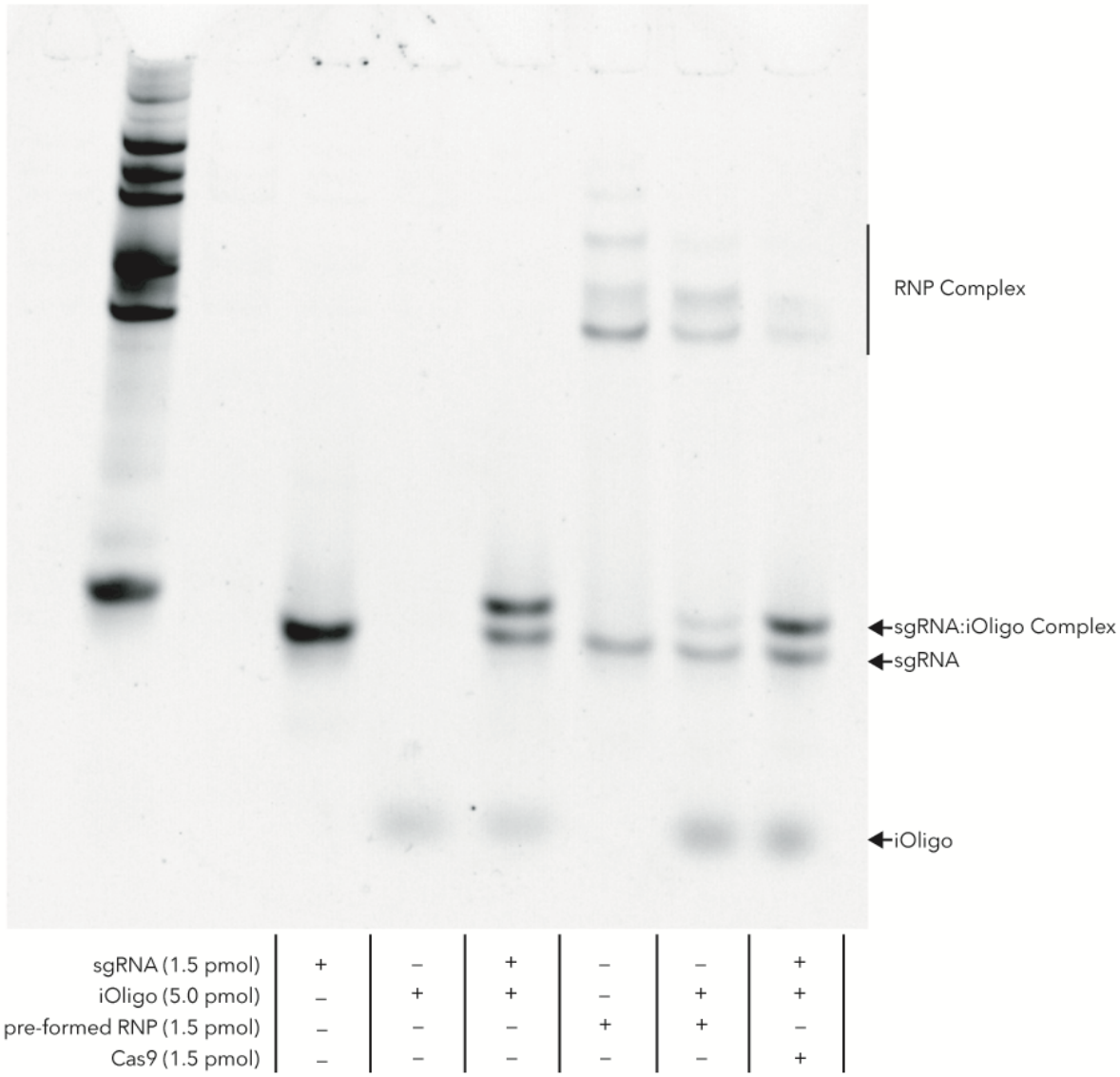
A, G, U, C: RNA nucleotide
a, g, u, c: 2'-O-Methyl nucleotide
s: phosphorothioate linkage

c



Supplementary Figure 1. (a) Sequences for iOligo variants A-D, corresponding to Figure 1. **(b)** Sequence and chemical modifications for sgICAM-2. **(c)** Normalized indel percentages as a function of time between iOligo treatment (first) and Cas9 mRNA + sgRNA (second) in iMAECs. *p<0.05, **p<0.01, one-way ANOVA. average +/- SEM.

a



Supplementary Figure 2. (a) EMSA showing complex formation between combinations sgRNA, Cas9 protein, and iOligo. 1.5 pmol of sgICAM-2 was mixed with and without 1.5 pmol spCas9 protein in the absence or presence of 5 pmol iOligo in the presence of an appropriate binding buffer. Complex formation was allowed to occur for 30 min at room temperature for either one or two stages before adding a loading dye. The samples were run on a 5% tris-borate EDTA (TBE) gel in 1X TBE electrophoresis buffer for 15 minutes. The gel was visualized with a SYBR Green EMSA nucleic acid gel stain. The upper shift bands in the protein present lanes might be due to the Cas9 nuclease forming dimers or multimers under the salt conditions of the binding buffer. This could be due to the C-terminal 6-His tag on the protein. It is also possible that under these conditions, the protein is following the wrong folding pathway, resulting in an alternative size or charge of the complex.

a

Figure	Mouse	Mice / Group	LNP-1	Cargo / Dose (mg / kg)	LNP-2	Cargo / Dose (mg / kg)	LNP-3	Cargo / Dose (mg / kg)
3C	Cas9	4	Hepat	iOligo / 1.2	Hepat	sgGFP / 0.5	-	-
3F	Cas9	4	Hepat	iOligo / 1.2	Lung	sgGFP / 1.0	-	-
3I	WT	4	Hepat	iOligo / 1.2	Spleen	sgGFP / 1.0	-	-
4F	WT	4	Hepat	siUTR / 1.0	Hepat	sgICAM-2 and Cas9 mRNA / 2.0 total	-	-
4H	WT	4	Hepat	siUTR / 1.0	Spleen	sgICAM-2 and Cas9 mRNA / 2.0 total	-	-
5B-D	WT	4	Hepat	siUTR / 1.0	Hepat	iOligo / 1.2	Spleen	sgICAM-2 and Cas9 mRNA / 2.0 total
6D-F	Cas9	3-4	Hepat	iOligo/ 1.2	Lung	sgICAM-2 / 2.0	-	-

b

LNP-Hepat		LNP-Lung		LNP-Spleen	
cKK-E12	50	7C1	80	7C1	60
Cholesterol	38.5	Cholesterol	0	Cholesterol	10
C14PEG2000	1.5	C14PEG2000	20	C14PEG2000	25
DSPC	10	Helper Lipid	0	DOPE	5
Lipid : RNA	7.5	Lipid : RNA	5	Lipid : RNA	10

C

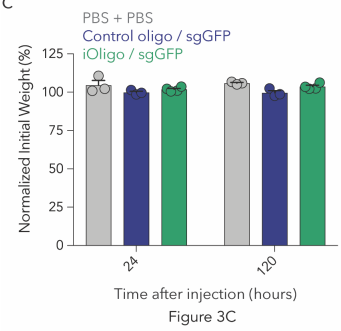


Figure 3C

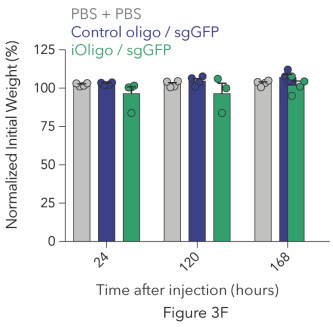


Figure 3F

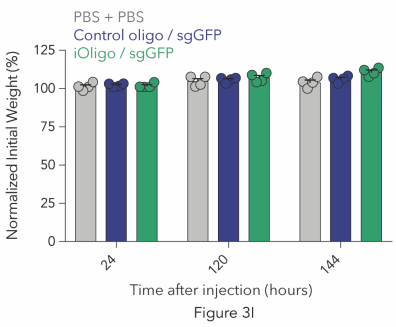


Figure 3I

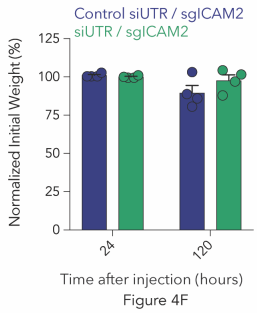


Figure 4F

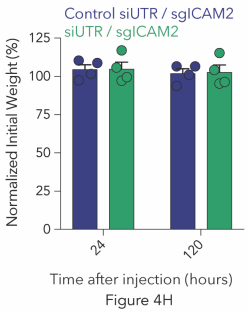


Figure 4H

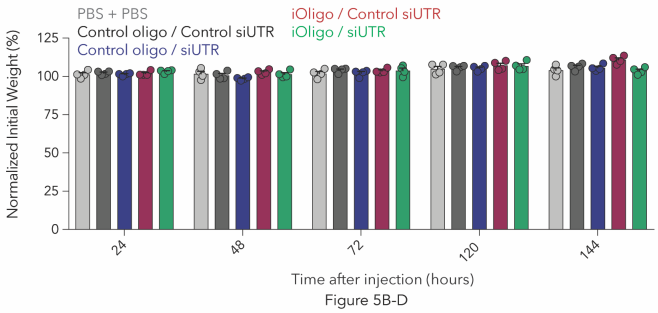


Figure 5B-D

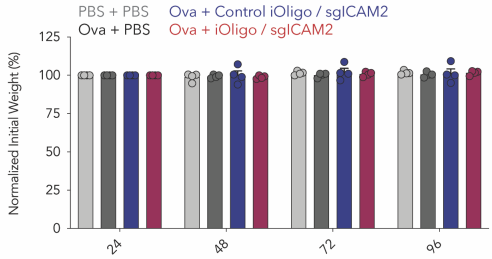


Figure 6A
LNP Pre-treatment

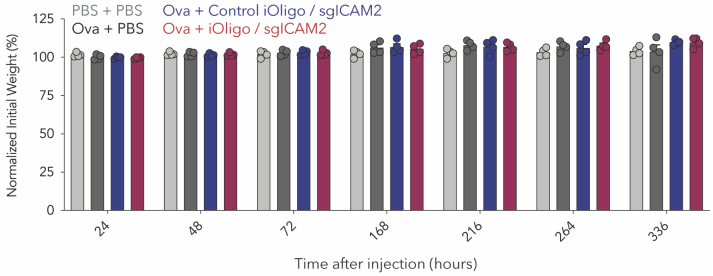
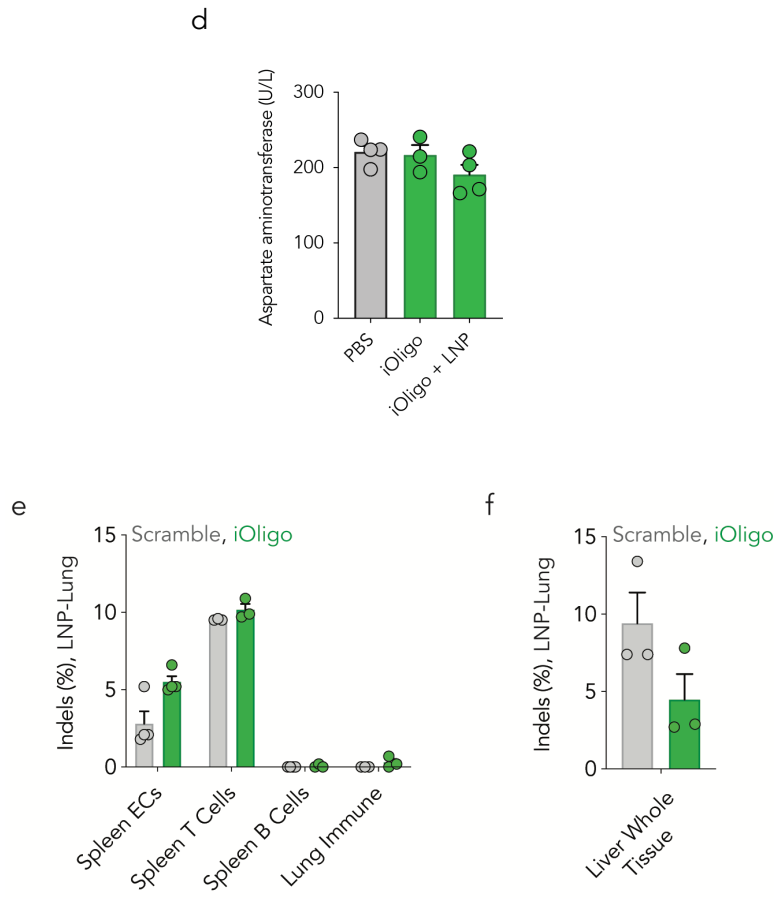


Figure 6B
Ova Treatment



Supplementary Figure 3. (a) Experimental details for all *in vivo* data sets, **(b)** including LNP component formulation ratios and lipid to RNA molar ratios. **(c)** Normalized mouse weights for all *in vivo* data sets. **(d)** Aspartate aminotransferase (AST) levels after administering 1X PBS, iOligo, or iOligo and LNP carrying sgRNA. We observed no measurable changes in AST levels. **(e)** Editing in spleen, lung cell types, and **(f)** whole liver with iOligo or control in LNP-Lung and **(g,h)** LNP-Spleen. average +/- SEM.

a

b

siICAM-2 sense: 5′ - AGGAcGGucucAAcuuuuc dT sdT - 3′
siICAM-2 antisense: 5′ - GAAAAGUuGAGACCGUCCU dT sdT -3′

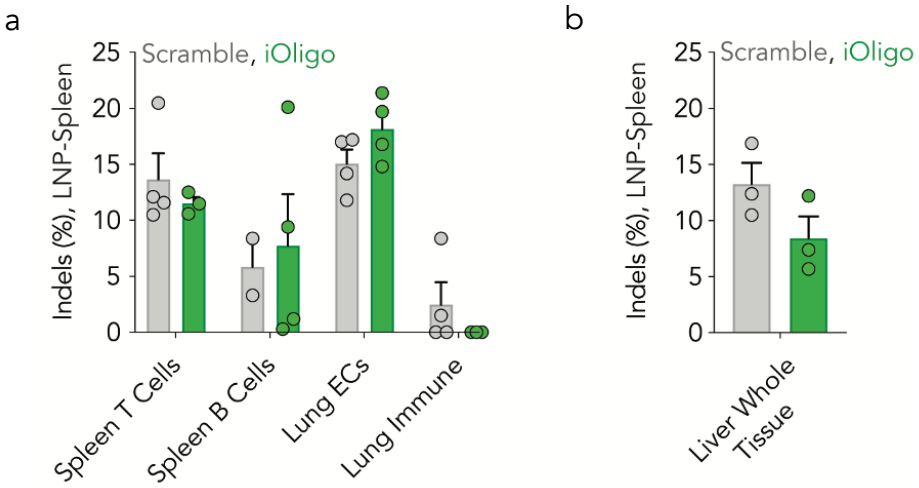
siControl sense: 5′ - cuuAcGcuGAGuAcuucGAdTsdT- 3′
siControl antisense: 5′ - UCGAAGuACUcAGCGuAAGdTsdT -3′

siGFP sense: 5′ - AcAuGAAGcAGcACGACuU dT sdT - 3′
siGFP antisense: 5′ - AAGUCGUGCUGCUUCAUGU dT sdT -3′

hm-siGFP sense: 5′ - Hexynyl- acAfuGfaAfgCfaGfcAfcGfaCfuUfdTsdT - 3′
hm-siGFP antisense: 5′ - AfAfgUfcGfuGfcUfgCfuUfcAfuGfudTsdT -3′

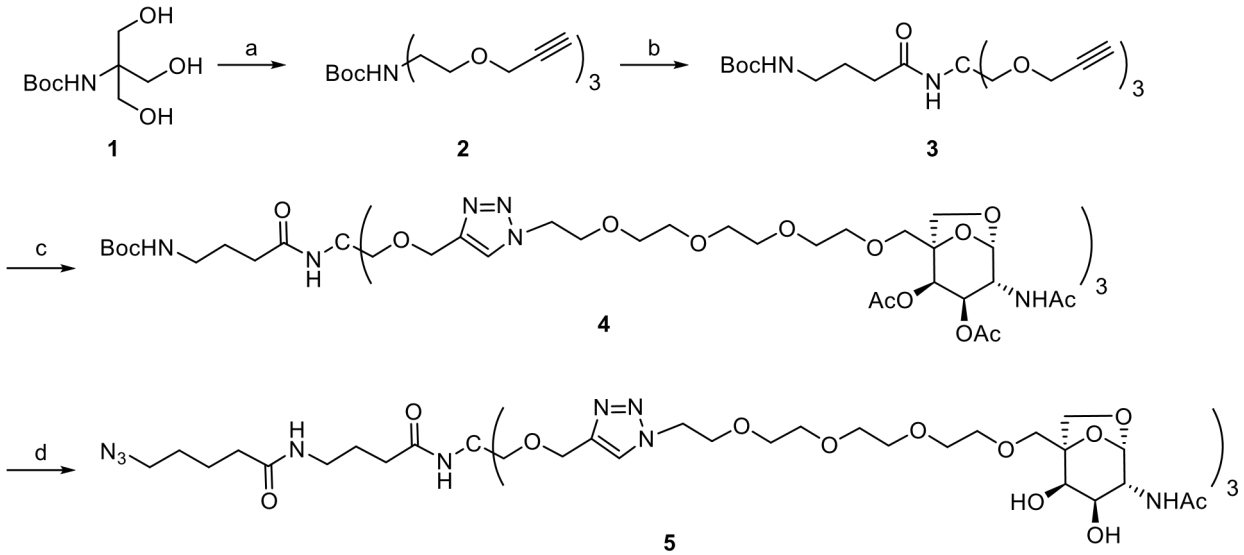
A, G, U, C: RNA nucleotide
dT: deoxy-T
a, g, u, c: 2′-O-Methyl nucleotide
s: phosphorothioate

Supplementary Figure 4. (a) Engineered 3' UTR with 5 siGFP-binding sites. **(b)** Sequence and chemical modifications of siRNAs used in this study.

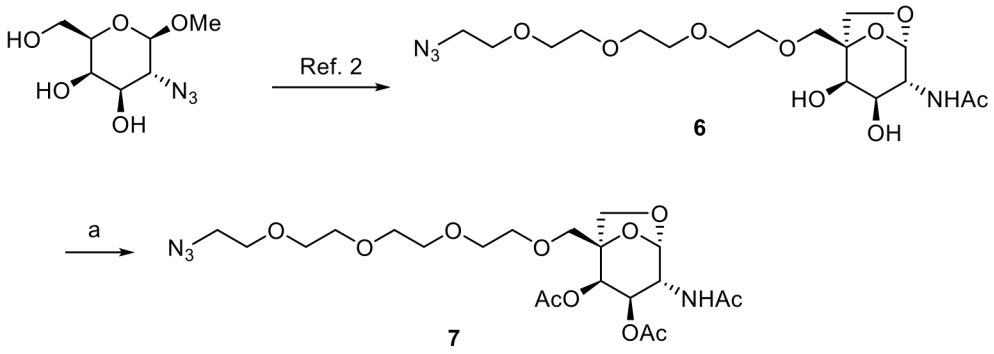


Supplementary Figure 5. (a) Editing in spleen, lung cell types, and **(b)** whole liver with iOligo or control in LNP-Spleen. average +/- SEM.

Supplementary Figure 6. Synthesis of ASGPR ligand (cpd **5**)



Scheme 1. Synthesis of trimeric asialoglycoprotein receptor ligand (compound **5**). a) propargyl bromide, KOH, TBAI, THF, r.t. 24h; b) i. TFA, ii. *N*-Boc-GABA, HCTU, *i*Pr_2NEt, DMAP, DMF, r.t., 2 h; c) cpd **7** (see Scheme 2), $CuSO_4$•$5H_2O$, Na Ascorbate, MeOH, r.t. 5 h; d) i. TFA, ii. *N*-hydroxysuccinimidyl 5-azidopentanoate, pyridine, DMF, r.t. 18 h; iii. NaOMe, MeOH.



Scheme 2. Synthesis of ASGPR-ligandOAc; a) Ac_2O, pyridine, CH_2Cl_2, r.t. 12 hours.
Abbreviations. r.t. = room temperature, DMAP = N,N-dimethylaminopyridine, GABA = aminobutyric acid, HCTU = O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid

Compound 2. *N*-BocTris (4.15 g, 18.8 mmol) and propargyl bromide (10.0 mL, 80% solution in toluene, 67.8 mmol) were dissolved in THF (75 mL) at room temperature. Potassium hydroxide (4.4 g, 78.6 mmol), potassium iodide (375 mg, 2.3 mmol) and tetrabutylammonium iodide (375 mg, 1.0 mmol) were then added and the resulting suspension was vigorously stirred at r.t. for 24 hours. The mixture was diluted with ethyl acetate (150 mL) and washed with water (2 x 150 mL) in a separation funnel. The organic layer was dried over sodium sulfate, filtered, and the volatiles were removed by rotary evaporation. The resulting material was purified by normal-phase flash chromatography eluting with a gradient of ethyl acetate/hexanes (5:95 to 1:4). Compound **2** (5.48 g, 16.3 mmol, 87%) was obtained as a yellowish oil. ^{1}H NMR spectra was consistent with data reported in the literature:[1] ^{1}H NMR (500 MHz, $CDCl_3$) : 4.18 (d, *J* = 2.4 Hz, 6H), 3.82 (s, 6H), 2.45 (dd, *J* = 2.4 and 2.4 Hz, 3H), 1.45 (s, 9H).

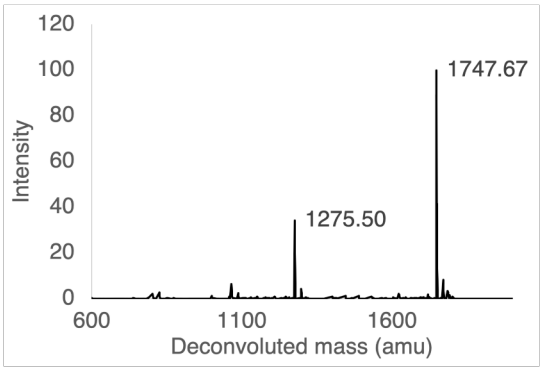
Compound 3: Alkynyl derivative **2** (1.74 g, 5.20 mmol) was subjected to *N*-Boc deprotection by treatment with trifluoroacetic acid (10.0 mL) for 15 minutes. The acid was then removed by rotary evaporation and the resulting oil was kept under high vacuum for 6 hours on a Schlenk line. The resulting syrup was dissolved in

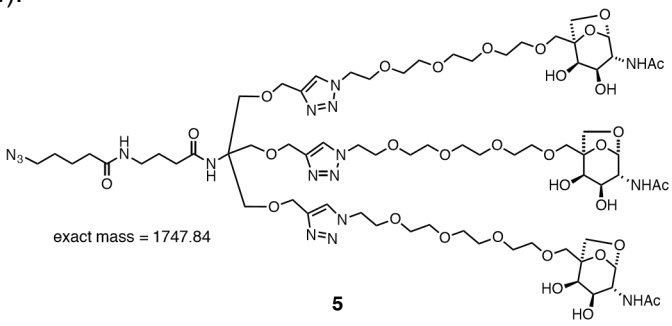
[1] . Chabre, Y.M.; Contino-Pépin, C.; Placide, V.; Shiao, T.C.; Roy, R. *J. Org. Chem.* **2008**, *73*, 5602-5605.

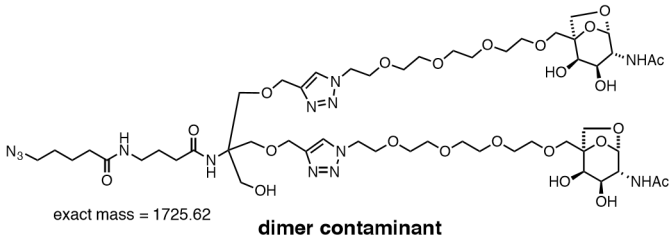
DMF (20.0 mL) together with *i*Pr_2NEt (2.7 mL, 15.5 mmol) and DMAP (63.5 mg, 0.52 mmol). The solution was stirred for 20 minutes at r.t. followed by addition of a freshly made solution of *N*-Boc-GABA (1.05 g, 5.2 mmol) and HCTU (2.58 g, 6.24 mmol) in DMF (10 mL). After 2 hours stirring at r.t., TLC showed complete consumption of the starting propargylic material and the mixture was diluted with EtOAc (100 mL), transferred to a separation funnel and washed sequentially with 1M HCl, saturated $NaHCO_3$, and brine. The organic layer was collected, dried over Na_2SO_4, filtered and the volatiles removed by rotary evaporation. The resulting material was purified by normal phase flash chromatography eluting with a gradient of EtOAc/hexanes (1:4 to 7:3). Compound **3** (1.04 g, 2.47 mmol, 48 %) was isolated as a yellowish oil. ^{1}H NMR (500 MHz, $CDCl_3$) : 4.14 (d, *J* = 2.4 Hz, 6H), 3.84 (s, 6H), 3.17 (m, 2H), 2.43 (dd, *J* = 2.4 and 2.4 Hz, 3H), 2.19 (dd, *J* = 7.1 and 7.1 Hz, 2H), 1.78 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) : 172.85, 156.3, 79.5, 74.8, 68.4, 60.3, 59.2, 58.6, 39.6, 34.3, 28.4, 26.1

Compound 4: Alkyne derivative **3** (33.6 mg, 0.080 mmol) and azide **7** (168.2 mg, 0.32 mmol) were dissolved in methanol (2.0 mL) at room temperature. A fresh aqueous solution of Cu(I) catalyst, prepared by dissolving $CuSO_4$ (23.9 mg, 0.096 mmol) and Na ascorbate (63.4 mg, 0.32 mmol) in water (0.5 mL), was added and the mixture was stirred at r.t. for 4 hours, capped to prevent access by air. Volatiles were then removed *in vacuo* and the obtained residue was purified by normal phase flash chromatography eluting with a gradient of MeOH /CH_2Cl_2 (1:99 to 1:9). Compound **4** (125.6 mg, 0.064 mmol, 80%) was obtained as a colorless syrup, confirmed by mass spectrometry.

Compound 5: *N*-Boc protected compound **4** (125.6 mg, 0.064 mmol) was treated with TFA (3.0 mL) for 15 minutes at r.t. The acid was then removed *in vacuo* and the resulting oil was kept under high vacuum for 6 hours in a Schlenk line. The crude material was then dissolved in a DMF/pyridine mixture (1.5 mL, 2:1) and stirred for 20 min at r.t. followed by the addition of *N*hydroxysuccinimidyl 5-azidopentanoate (46.1 mg, 0.192 mmol). Stirring was continued for 24 hours, after which the volatiles were then removed *in vacuo*. The crude product was purified by normal phase flash chromatography eluting with a gradient of MeOH/CH_2Cl_2 (1:99 to 1:4). The resulting material was dissolved in dry MeOH (3.0 mL) and treated with NaOMe (~3.0 mg). This acetate deprotection reaction was stirred for 48 h at room temperature. The mixture was then neutralized with Amberlite IR 120 (H form), filtered, and the solvent was removed by rotary evaporation. Compound **5** (55.9 mg, 0.032, 50%) was obtained as a colorless oil, shown to contain a small amount of a dimer contaminant by mass spectrometry (shown below).





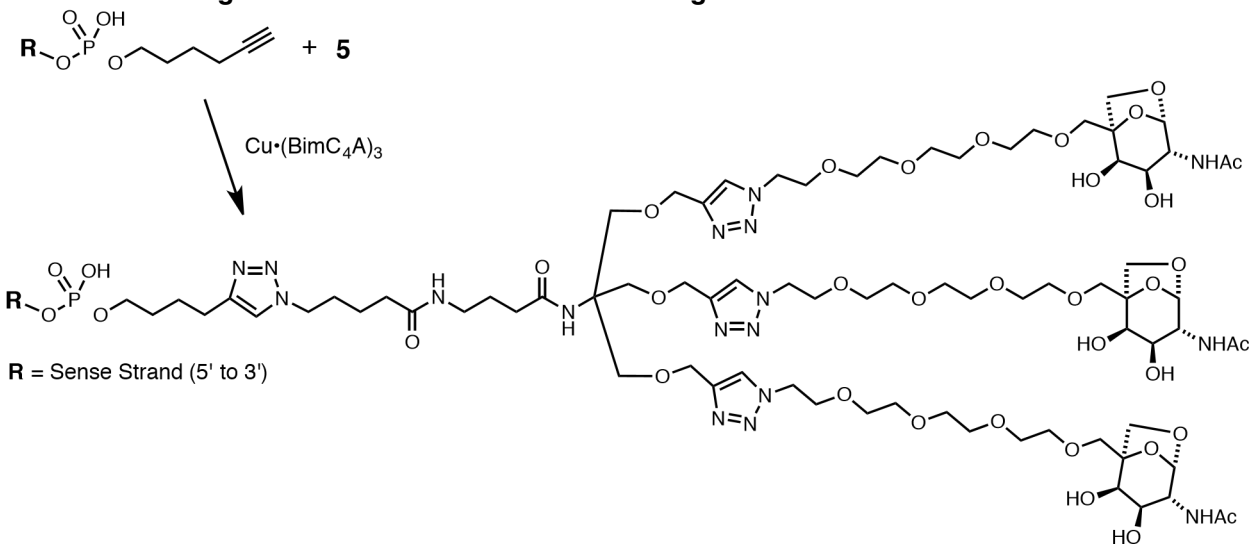


a) High-resolution ESI-LC-TOF analysis of ligand **5**. The lower-mass peak is due to the presence of a small amount of the dimer structure shown, also a strong binder of ASGPR.[2]

[2] . Sanhueza, C.A.; Baksh, M.M.; Thuma, B.; Roy, M.R.; Dutta, S.; Preville, C.; Chrunyk, B.A.; Beaumont, K.; Dullea, R.; Ammirrati, M.; Liu, S.; Gebhard, D.; Finley, J.; Salatto, C.T.; King-Ahmad, A.; Stock, I.; Atkinson, K.; Reidich, B.; Lin, W.; Kumar, K.; Tu, M.; Menhaji-Klotz, E.; Price, D.A.; Liras, S.; Finn, M.G.; Mascitti, V. *J. Am. Chem. Soc*. **2017**, *139*, 3528-3536.

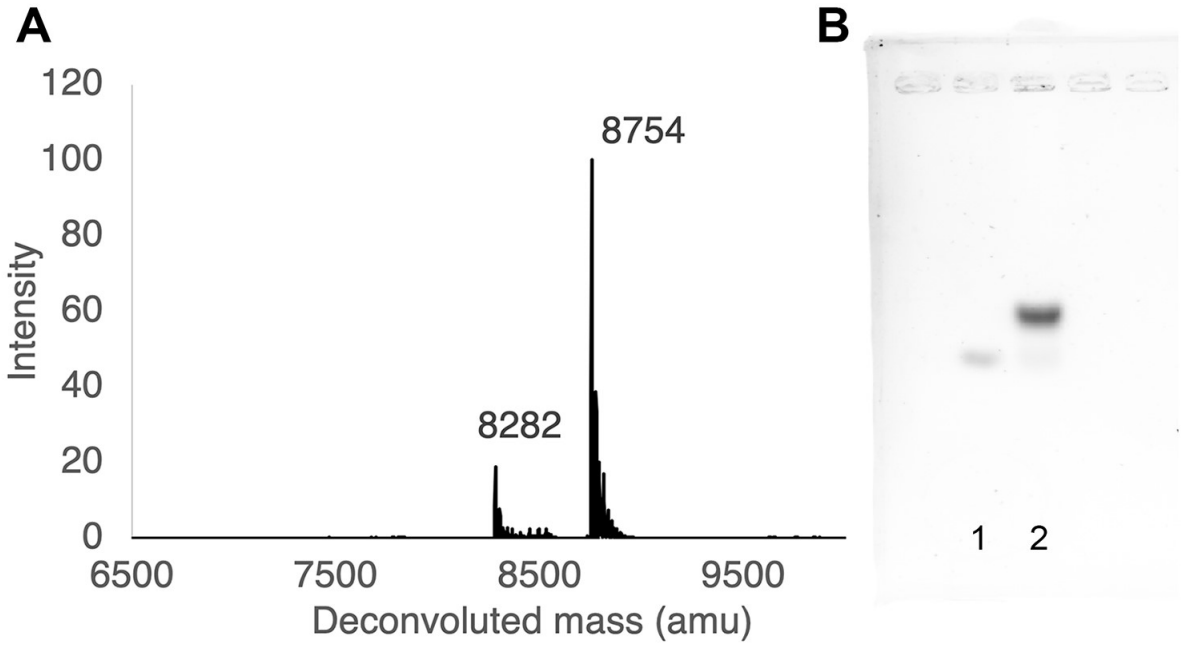
Compound 6: ASGPR-ligand **6**[2] (454.6 mg, 1.04 mmol) was dissolved in dry CH_2Cl_2 (5.0 mL) and treated with acetic anhydride (0.8 mL, 8.32 mmol) and pyridine (0.7 mL, 8.32 mmol). The resulting solution was stirred at r.t. for 16 hours. After this time, TLC showed complete consumption of the starting diol and the appearance of a single species. Volatiles were removed by rotary evaporation and the resulting material was purified by normal phase flash chromatography, eluting with a gradient of MeOH/CH_2Cl_2 (1:99 to 1:9). Compound **7** (490.4 mg, 0.94 mmol) was isolated as a colorless syrup.

Procedure for oligonucleotide attachment to ASGPR ligands

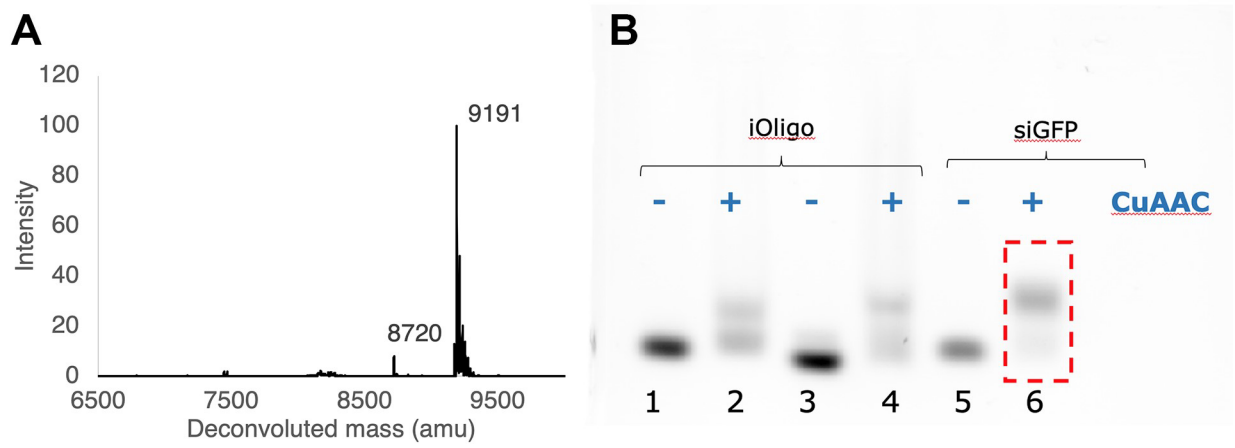


To a solution of siGFP-alkyne or iOligo-alkyne (1.56 μmol) in nuclease-free water was added a mixture of copper sulfate (8.0 equiv relative to alkyne, 12.56 μmol, 2.51 μL from a 500 mM stock in water), the copper-binding ligand $(BimC_4A)_3$[3] (8.0 equiv, 12.56 μmol, 5.02 μL from a 250 mM stock in DMSO), compound **5** (1.25 equiv, 1.96 μmol, 9.81 μL from a 20 mM stock in DMSO), and aminoguanidine (220 μmol, 22 μL from a 1 M stock in water). A freshly prepared solution of sodium ascorbate (220 μmol, 22 μL from a 1 M stock in water) was added, and the reaction mixture was gently inverted to mix and incubated at 50 °C for one hour. The conjugates were purified by Zeba 7k MW desalting column, used according to the manufacturer's instructions, followed by ethanol precipitation with three volumes of EtOH, onetenth volume of 3 M sodium acetate, pH 5.2, and 10 μg glycogen. The extent of reaction and conjugate purity were determined by electrophoretic analysis and by high resolution ESI-TOF MS.

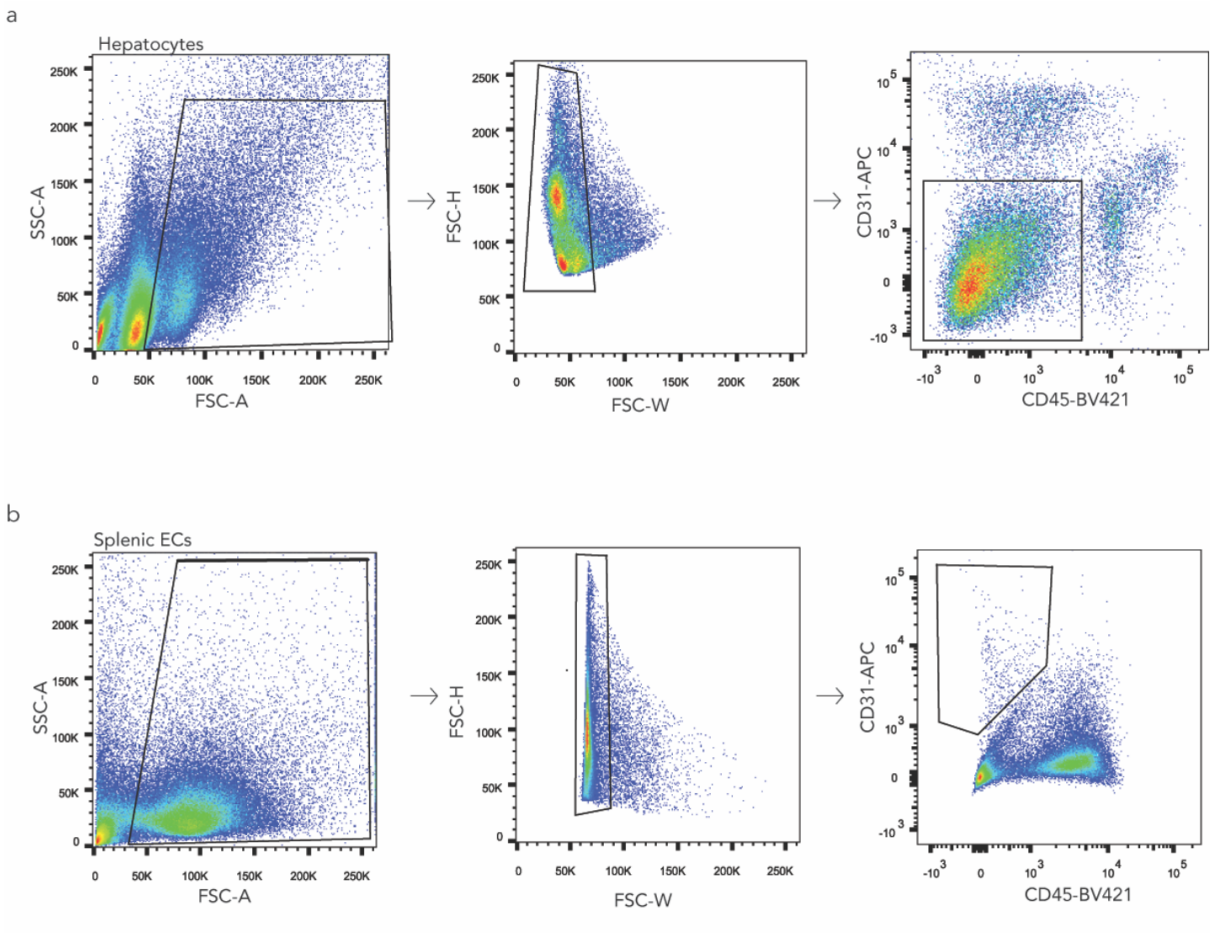
[3] . Rodionov, V.O.; Presolski, S.; Gardinier, S.; Lim, Y.-H.; Finn, M.G. *J. Am. Chem. Soc.* **2007**, *129*, 12696-12704. Available from Sigma-Aldrich (catalog number 696854).



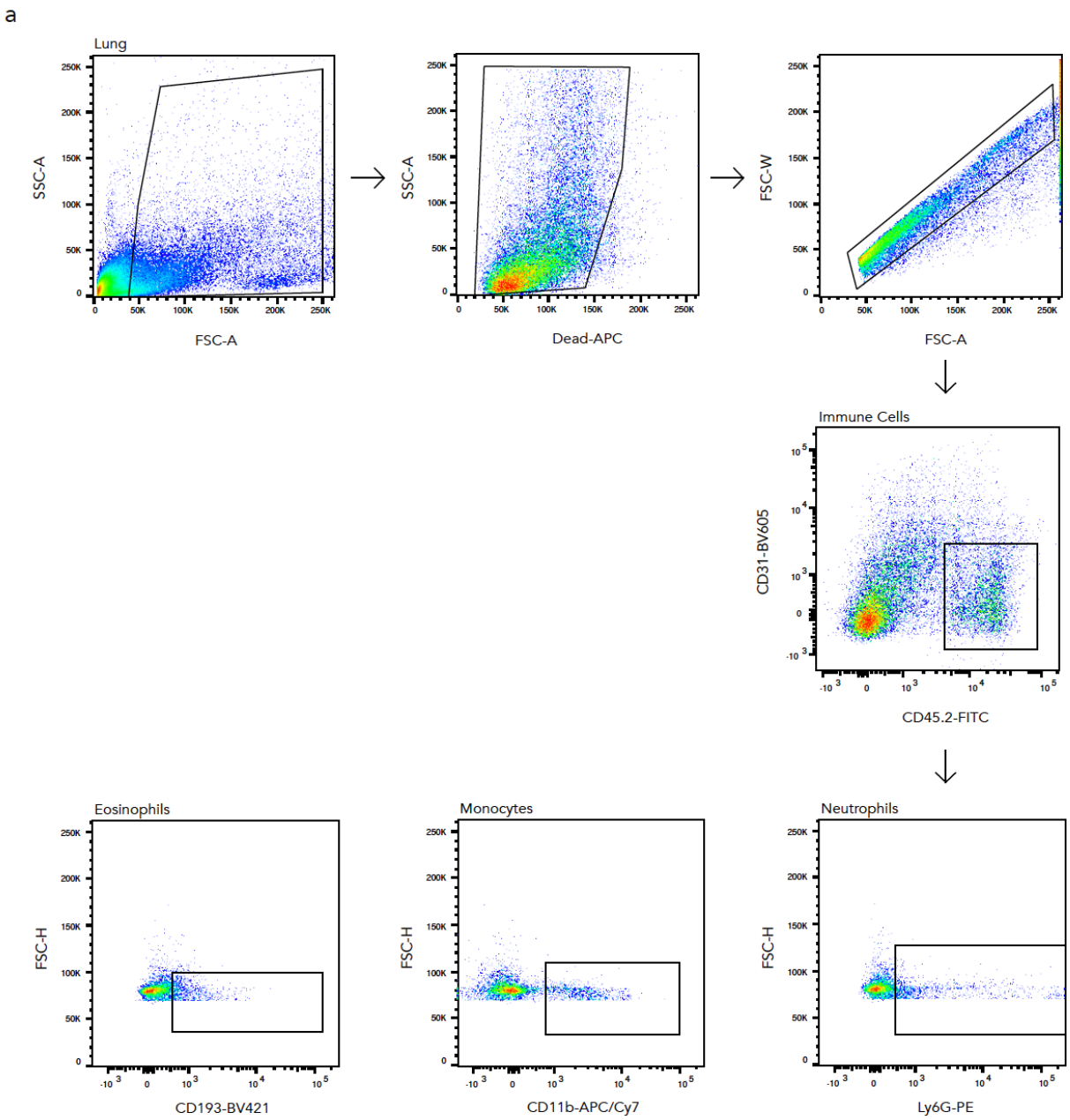
Supplementary Figure 7. (A) High-resolution ESI-LC-TOF analysis of siGFP conjugated to the ASGPRL trimer (5). The smaller peak is due to conjugation to the dimeric contaminant of 5. (B) Agarose gel electrophoresis of siGFP (lane 1) and the siGFP-ASGPRL conjugate (lane 2), showing approx. 90% conversion to the product after a one-hour reaction.



Supplementary Figure 8. **(A)** High-resolution ESI-LC-TOF analysis of **iOligo** conjugated to the ASGPRL trimer (**5**). The smaller peak is due to conjugation to the dimeric contaminant of **5**. **(B)** Agarose gel electrophoresis of iOligo and siGFP conjugation reactions to ASGPR ligand, loading approx. 500 ng of nucleic acid per lane, without purification, after 1 h reaction time. Lanes 1, 3, 5 = starting oligonucleotides; Lanes 2, 4, 6 = reaction mixtures.



Supplementary Figure 9. Representative FACS gating for **(a)** liver hepatocytes and **(b)** splenic endothelial cells.



Supplementary Figure 10. (a) Representative FACS gating for eosinophils, monocytes, and neutrophils.