Electronic Supplementary Material (ESI) for Chemical Science.
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Supplementary Information

NKT Cell-Dependent Glycolipid-Peptide Vaccines with Potent Anti-tumor Activity

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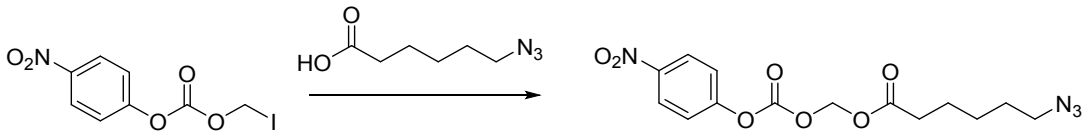
General chemical synthesis methods. Anhydrous solvents were obtained commercially. Solvents required for oxime ligation coupling reactions were distilled from 2,4-dinitrophenylhydrazine immediately prior to use. Air-sensitive reactions were carried out under Ar. Thin layer chromatography (TLC) was performed on aluminium sheets coated with 60 F_{254} silica. Flash column chromatography was performed on Reveleris® silica cartridges (38.6 µm) or SiliCycle® silica gel (40 - 63 µm). NMR spectra were recorded on a Bruker 500 MHz spectrometer. ^{1}H NMR spectra were referenced to tetramethylsilane at 0 ppm (internal standard) or to residual solvent peak ($CHCl_3$ 7.26 ppm, CHD_2OD 3.31 ppm, $CHD_2(SO)CD_3$ 2.50 ppm). ^{13}C NMR spectra were referenced to tetramethylsilane at 0 ppm (internal standard) or to the deuterated solvent peak ($CDCl_3$ 77.0 ppm, CD_3OD 49.0 ppm, $(CD_3)_2SO$ 39.5 ppm). $CDCl_3$-CD_3OD solvent mixtures were always referenced to the methanol peak. High resolution electrospray ionization (ESI) mass spectra and HPLC-ESI-MSMS analyses were undertaken on a Waters Q-TOF Premier™ Tandem Mass spectrometer fitted with a Waters 2795 HPLC. Semi-preparative HPLC and synthetic purity HPLC data were obtained on an Agilent 1100 system and peak identity was confirmed by LCMS on an Agilent 1260 HPLC with an Agilent 6130 single quadrupole mass spectroscopic detector using ESI. Each of these latter two systems was coupled to a Dionex Corona Ultra RS charged aerosol detector (CAD) as required. Fmoc-L-Leu-O-CH2-C6H4-OCH2-CH2-COOH linker was purchased from PolyPeptide Group (Strasbourg, France). Nα-9-Fluorenylmethoxycarbonyl (Fmoc) protected L-α-amino acids and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were purchased from GL Biochem (Shanghai, China). Dimethylformamide (DMF) and acetonitrile were purchased from Global Science (Auckland, NZ); 3,6-dioxa-1,8-octanedithiol (DODT), triisopropylsilane (TIPS), diisopropylethylamine (DIPEA), and piperidine were purchased from Sigma Aldrich (St Louis, MO). Trifluoroacetic acid (TFA) was obtained from Oakwood Products, Inc (West Colombia, SC).

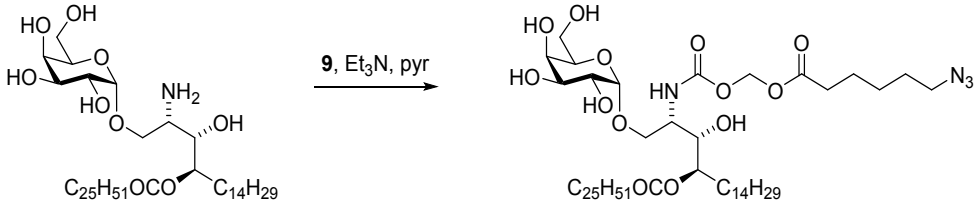
(((4-Nitrophenoxy)carbonyl)oxy)methyl 6-azidohexanoate 9



8 **9**

A mixture of iodomethyl 4-nitrophenyl carbonate[1] (**8**) (340 mg, 1.05 mmol), 6-azidohexanoic acid (210 mg, 1.34 mmol), silver oxide (100 mg, 0.43 mmol) and 4Å molecular sieves in dry acetonitrile (5 mL) was protected from light and stirred at rt. After 24 h, the mixture was filtered through celite, washed with EtOAc (20 mL) and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (EtOAc/toluene 0:10 to 1:4) to afford the title compound **9** as a colourless oil (150 mg, 40%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42-1.48 (m, 2 H),1.60-1.66 (m, 2 H), 1.68-1.74 (m, 2 H), 2.45 (dd, *J* = 7.4, 7.4 Hz, 2 H), 3.28 (dd, 6.8, 6.8 Hz, 2 H), 5.88 (s, 2 H), 7.41 (dd, J = 2.2, 9.2 Hz, 2 H), 8.29 (dd, *J* = 2.2, 9.2 Hz, 2 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 23.9, 26.0, 28.4, 33.5, 51.6, 82.5, 121.6, 125.3, 145.6, 151.4, 155.0, 171.6; HRMS-ESI: *m/z* calcd for $C_{14}H_{16}N_4O_7Na$ $[M+Na]^+$ 375.0917, found 375.0917.

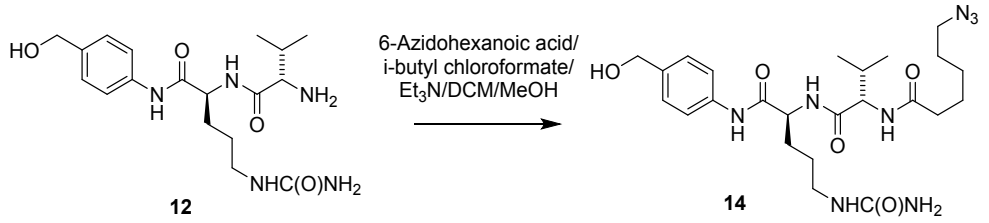
(2S,3S,4R)-2-(((((6-azidohexanoyl)oxy)methoxy)carbonyl)amino)-1-(α-D-galactopyranosyloxy)-3-hydroxyoctadecan-4-yl hexacosanoate (**11**)



1 **11**

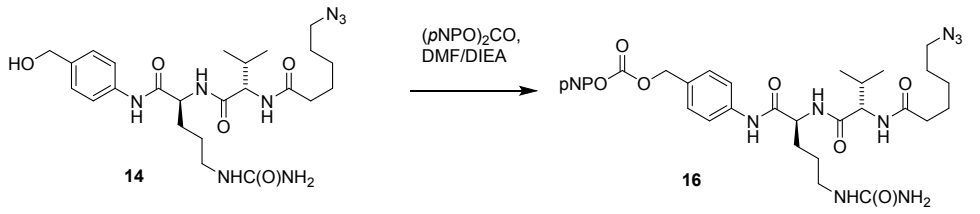
To a solution of amine **1** (25 mg, 0.029 mmol) in dry pyridine (1 mL) was added a solution of (4-nitrophenoxy)carbonyloxymethyl 6-azidohexanoate (**9**) (20 mg, 0.056 mmol) in CH_2Cl_2 (0.15 mL) followed by dry Et_3N (1 mL). After 0.5 h at rt, the mixture was diluted with MeOH and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (MeOH/$CHCl_3$ 0:10 to 2:8) to afford the title compound **11** as a white solid (21 mg, 67%). ^{1}H NMR (500 MHz, 3:1 $CDCl_3$/CD_3OD) δ 0.87-0.90 (m, 6 H), 1.23-1.35 (m, 68 H), 1.40-1.46 (m, 2 H), 1.60-1.71 (m, 8 H), 2.33-2.37 (m, 2 H), 2.40 (dd, *J* = 7.5, 7.5 Hz, 2 H), 3.29 (dd, *J* = 6.7, 6.7 Hz, 2 H), 3.72-3.80 (m, 8 H), 3.87 (dd, *J* = 2.3, 10.3 Hz, 1 H), 3.96 (d, *J* = 2.9 Hz, 1 H), 4.86 (d, *J* = 3.7 Hz, 1 H), 4.91-4.94 (m, 1 H), 5.73 (s, 2 H), 6.78 (d, *J* = 8.7 Hz, 1 H); ^{13}C NMR (126 MHz, 3:1 $CDCl_3$/CD_3OD) δ 14.2, 22.9, 24.3, 25.3, 25.6, 26.3, 28.7, 29.0, 29.4, 29.6, 29.7, 29.8, 29.9, 32.2, 34.0, 34.8, 51.4, 52.4, 62.1, 68.1, 69.3, 70.1, 70.5, 70.9, 71.9, 74.9, 80.4, 100.1, 155.1, 173.1, 174.8; HRMS-ESI: *m/z* calcd for $C_{58}H_{110}N_4O_{13}Na$ $[M+Na]^+$ 1093.7967, found 1093.7972.

***N*-(6-Azidohexanoyl)-Val-Cit-4-aminobenzyl alcohol (14)**



To a stirred solution of 6-azidohexanoic acid (85.0 mg, 0.541 mmol) in anhydrous CH_2Cl_2 (3.3 mL) at 0 °C was added by Et_3N (80 µL, 0.57 mmol), followed by isobutyl chloroformate (68 µL, 0.52 mmol). After 30 min, the solution was transferred by cannula to a separate flask containing amine **12**[2] (166 mg, 0.438 mmol) dissolved in 3:1 CH_2Cl_2-MeOH (4 mL) at 0 °C. The original flask was rinsed with CH_2Cl_2 (2 x 0.5 mL), which was transferred to the second flask. After 5 min, the reaction mixture was warmed to rt and stirred for 2.5 h. After concentration of the solvents under reduced pressure, the resulting solid was triturated successively with toluene, diethyl ether, acetone and MeCN, and purified by column chromatography on silica gel (MeOH/$CHCl_3$ = 10:90 to 14:86) to afford the title compound **14** as a white solid (160 mg, 71%). ^{1}H NMR (500 MHz, 2:1 $CDCl_3$/CD_3OD) δ 0.95-97 (m, 6 H), 1.39-1.45 (m, 2 H), 1.53-1.77 (m, 7 H), 1.88-1.95 (m, 1 H), 2.04-2.11 (m, 1 H), 2.29 (t, *J* = 7.5 Hz, 2 H), 3.09-3.15, (m, 1 H), 3.20-3.26 (1 H), 3.28 (t, *J* = 6.9 Hz, 2 H), 4.19, (d, *J* = 7.3 Hz, 1 H), 4.54 (dd, *J* = 5.0, 8.8 Hz, 1 H), 4.59 (s, 2 H), 7.31 (d, *J* = 8.5 Hz, 2 H), 7.54 (d, *J* = 8.5 Hz, 2 H); ^{13}C NMR (126 MHz, 2:1 $CDCl_3$/CD_3OD) δ 18.4, 19.4, 25.6, 26.6, 28.9, 29.6, 31.0, 36.2, 39.4, 51.6, 53.6, 59.4, 64.3, 120.5, 127.9, 137.4, 137.7, 161.0, 170.9, 172.8, 174.9; HRMS-ESI $[M+Na]^+$ calcd for $C_{24}H_{38}N_8NaO_5$: 541.2863; found 541.2860.

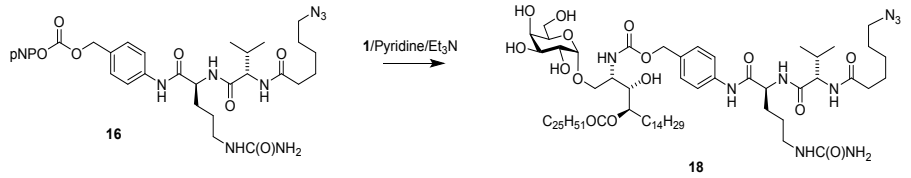
***N*-(6-Azidohexanoyl)-Val-Cit-4-aminobenzyl 4-nitrophenyl carbonate (16)**



To a mixture of alcohol **14** (158 mg, 0.305 mmol) in anhydrous DMF (2.5 mL) was added *N,N*-diisopropylethylamine (66 µL, 0.38 mmol) followed by bis(4-nitrophenyl) carbonate (116 mg, 0.381 mmol) and the reaction was stirred under Ar at rt for 41 h. After concentrating the mixture under high vacuum, the crude product was purified by column chromatography on silica gel (MeOH/CH_2Cl_2 = 6:94 to 11:89) to afford the title compound **16** as an off-white solid (206 mg, 99%). ^{1}H NMR (500 MHz, d6-DMSO) δ 0.84 (d, *J* = 6.8 Hz, 3 H), 0.87 (d, *J* = 6.7 Hz, 3 H), 1.27-1.33 (m, 2 H), 1.34-1.64 (m, 7 H), 1.68-1.75 (m, 1 H), 1.95-2.02 (m, 1 H), 2.13-2.24 (m, 2 H), 2.92-2.98 (m, 1 H), 3.00-3.06, (m, 1 H), 4.18-4.21 (m, 1 H), 4.38-4.42 (m, 1 H), 5.24 (s, 2 H), 5.39 (s, 2 H), 5.96 (t, *J* = 5.7 Hz, 1 H), 7.41 (d, *J* = 8.4 Hz, 2 H), 7.55-7.58 (m, 2 H), 7.65 (d, *J* = 8.4 Hz, 2 H), 7.81 (d, *J* = 8.6 Hz, 1 H), 8.06 (d, *J* = 7.5 Hz, 1 H), 8.29-8.33 (m, 2 H), 10.03 (s, 1 H); ^{13}C NMR (126 MHz, d6-DMSO) δ 18.2, 19.2, 24.8, 25.7, 26.8,

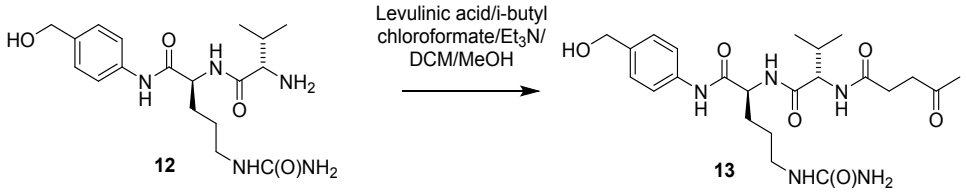
27.9, 29.2, 30.3, 34.9, 38.5, 50.5, 53.1, 57.6, 70.2, 119.0, 122.5, 125.3, 129.3, 129.4, 139.3, 145.1, 151.9, 155.3, 158.8, 170.7, 171.3, 172.3; HRMS-ESI $[M+Na]^+$ calcd for $C_{31}H_{41}N_9NaO_9$: 706.2925; found 706.2913.

2*S*,3*S*,4*R*)-2-(*N*-(6-Azidohexanoyl)-Val-Cit-4-aminobenzyloxycarbonylamino)-(*N*-Val-Cit-4-aminobenzyloxycarbonylamino)-1-(α-D-galactopyranosyloxy)-3-hydroxy-octadecan-4-yl hexacosanoate (18)



To a mixture of **1** (61 mg, 0.071 mmol) and pNP-carbonate **16** (54 mg, 0.079 mmol) in anhydrous pyridine (1.0 mL) under Ar was added Et_3N (20 µL, 0.14 mmol) and the mixture was stirred at rt. After 26 h, the mixture was concentrated to dryness under high vacuum, and the crude residue was purified by column chromatography on silica gel ($MeOH/CH_2Cl_2$ = 5:95 to 20:80), followed by column chromatography on C18 silica gel ($MeOH/CH_2Cl_2$ = 100:0 to 90:10), to afford the title compound **18** as a white solid (57 mg, 57%). ^1H NMR (500 MHz, 2:1 $CDCl_3/CD_3OD$) δ 0.87-0.90 (m, 6 H), 0.95-0.98 (m, 6 H), 1.24-1.37 (m, 68 H), 1.39-1.45 (m, 2 H), 1.53-1.77 (m, 11 H), 1.87-1.94 (m, 1 H), 2.04-2.11 (m, 1 H), 2.27-2.32 (m, 2 H), 2.33-2.40 (m, 2 H), 3.09-3.14 (m, 1 H), 3.21-3.26 (m, 1 H), 3.28 (t, *J* = 6.8 Hz, 2 H), 3.66-3.80 (m, 8 H), 3.85-3.87 (m, 2 H), 4.18 (d, *J* = 7.3 Hz, 1 H), 4.53 (dd, *J* = 5.1, 8.6 Hz, 1 H), 4.85 (d, *J* = 3.7 Hz, 1 H), 4.93-4.99 (m, 2 H), 5.10-5.18 (m, 1 H), 7.32 (d, *J* = 8.3 Hz, 2 H), 7.57 (d, *J* = 8.3 Hz, 2 H); ^{13}C NMR (126 MHz, 2:1 $CDCl_3/CD_3OD$) δ 14.2, 18.5, 19.4, 23.0, 25.4, 25.6, 25.7, 26.7, 28.9, 29.2, 29.6, 29.69, 29.72, 29.8, 29.90, 29.92, 29.96, 30.02, 30.06, 31.0, 32.3, 35.0, 36.2, 39.4, 51.6, 52.6, 53.7, 59.4, 62.3, 66.8, 68.4, 69.4, 70.2, 70.7, 71.0, 72.3, 75.1, 100.4, 120.5, 129.1, 133.0, 138.3, 157.1, 161.1, 171.0, 172.9, 175.0; HRMS-ESI $[M+Na]^+$ calcd for $C_{75}H_{135}N_9NaO_{15}$: 1424.9975; found 1424.9940.

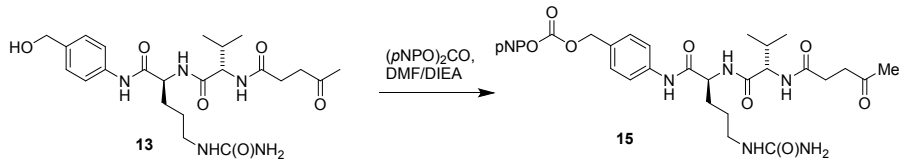
***N*-Levulinoyl-Val-Cit-4-aminobenzyl alcohol (13)**



To a stirred solution of levulinic acid (40 mg, 0.34 mmol) in anhydrous CH_2Cl_2 (2.0 mL) at 0 °C was added Et_3N (50 µL, 0.36 mmol), followed by isobutyl chloroformate (43 µL, 0.33 mmol). The solution was warmed to rt and stirred for 45 min, before transferring by cannula to a separate flask containing amine **12**[2] (100 mg, 0.264 mmol) in 5:1 CH_2Cl_2-MeOH (2.4 mL) at 0 °C. The original flask was rinsed with CH_2Cl_2 (0.5 mL), which was transferred to the second flask. After 5 min, the reaction mixture was warmed to rt and MeOH (1 mL) was added to aid stirring of the heterogeneous mixture. After 85 min at rt, the reaction was quenched with Et_2NH (25 µL) and the solvents were concentrated under reduced pressure. The resulting solid was triturated successively with diethyl ether and CH_2Cl_2, and purified by

column chromatography on silica gel ($MeOH/CH_2Cl_2$ = 10:90 to 18:82) to afford the title compound **13** as a white solid (94 mg, 75%). ^1H NMR (500 MHz, d6-DMSO) δ 0.84 (d, *J* = 6.8 Hz, 3 H), 0.86 (d, *J* = 6.8 Hz, 3 H), 1.32-1.48 (m, 2 H), 1.56-1.63 (m, 1 H), 1.68-1.75 (m, 1 H), 1.94-2.03 (m, 1 H), 2.07 (s, 3 H), 2.35-2.46 (m, 2 H), 2.59-2.70 (m, 2 H), 2.91-2.98 (m, 1 H), 2.99-3.05, (m, 1 H), 4.16 (dd, *J* = 6.6, 8.4 Hz, 1 H), 4.35-4.39 (m, 1 H), 4.43 (d, *J* = 5.7 Hz, 2 H), 5.07 (t, *J* = 5.7 Hz, 1 H), 5.38 (s, 2 H), 5.95 (t, *J* = 5.7 Hz, 1 H), 7.23 (d, *J* = 8.4 Hz, 2 H), 7.54 (d, *J* = 8.4 Hz, 2 H), 7.88 (d, *J* = 8.4 Hz, 1 H), 7.98 (d, *J* = 7.7 Hz, 1 H), 9.79 (s, 1 H); ^{13}C NMR (126 MHz, d6-DMSO) δ 18.1, 19.2, 26.8, 29.0, 29.3, 29.6, 30.3, 38.1, 38.6 53.1, 57.8, 62.6, 118.8, 126.9, 137.4, 137.5, 158.8, 170.3, 171.1, 171.7, 207.5; HRMS-ESI $[M+Na]^+$ calcd for $C_{23}H_{35}N_5NaO_6$: 500.2485; found 500.2485.

***N*-Levulinoyl-Val-Cit-4-aminobenzyl 4-nitrophenyl carbonate (15)**



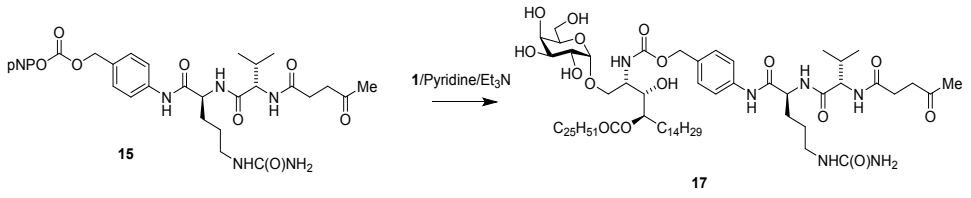
To a solution of alcohol **13** (89 mg, 0.19 mmol) in anhydrous DMF (1.7 mL) was added bis(4-nitrophenyl) carbonate (67 mg, 0.22 mmol) followed by *N,N*-diisopropylethylamine (39 µL, 0.22 mmol) and the reaction was stirred under Ar at rt for 7 h. The product was precipitated by the addition of diethyl ether and filtered, washing with diethyl ether and CH_2Cl_2. The crude product was purified by column chromatography on silica gel ($MeOH/CH_2Cl_2$ = 4:96 to 8:92) to afford the title compound **15** as a white solid (70 mg, 58%). ^1H NMR (500 MHz, 2:3 $CDCl_3/CD_3OD$) δ 1.00-1.03 (m, 6 H), 1.53-1.69 (m, 2 H), 1.78-1.86 (m, 1 H), 1.98-2.05 (m, 1 H), 2.08 (s, 3 H), 2.15-2.23 (m, 1 H), 2.44-2.50 (m, 1 H), 2.61 (ddd, *J* = 5.1, 8.7, 15.6 Hz, 1 H), 2.76-2.82 (m, 1 H), 2.88 (ddd, *J* = 5.4, 8.7, 18.6 Hz, 1 H), 3.13-3.23 (m, 1 H), 4.16 (d, *J* = 6.1 Hz, 1 H), 4.52 (dd, *J* = 4.7, 9.7 Hz, 1 H), 5.27 (s, 2 H), 7.40-7.44 (m, 4 H), 7.69 (d, *J* = 8.6 Hz, 2 H), 8.21-8.31 (m, 2 H); ^{13}C NMR (126 MHz, 2:3 $CDCl_3/CD_3OD$) δ 18.3, 19.5, 27.3, 29.6, 29.76, 29.83, 30.7, 39.0, 39.9, 54.4, 60.6, 71.3, 120.9, 122.7, 125.9, 130.1, 131.1, 139.6, 146.3, 153.4, 156.5, 161.5, 171.7, 173.3, 175.2, 210.3; HRMS-ESI $[M+Na]^+$ calcd for $C_{30}H_{38}N_6NaO_{10}$: 665.2547; found 665.2553.

***N*-(Aminooxyacetyl)-FFRKSIINFEKL (19)**

Peptide **19** was synthesised by Fmoc solid phase peptide synthesis on an aminomethyl polystyrene resin (manufactured in-house)[3] from Bio-Beads (Bio-Rad Laboratories, Inc, USA). Where necessary Nα-amino acids with the following protecting groups were used: Arg(Pbf), Lys(tBu), Ser(tBu), Asn(Trt) and the peptide was synthesised using a CEM Liberty microwave peptide synthesiser (AI Scientific, Queensland, Australia). The synthesis was conducted on a 0.25 mmol scale using reaction cycles consisting of Nα-Fmoc deprotection with 20% piperidine in DMF (30 seconds and then 3 minutes, 75 °C, 60 W) and couplings (5 minutes, 73 °C, 25 W) employing a 4-fold molar excess of the protected

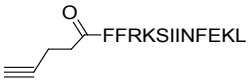
amino acid in DMF activated by a 3.6 molar excess of HCTU in the presence of an 8 molar excess of DIPEA. Nα-Fmoc-Arg(Pbf) was coupled for 25 minutes at room temperature followed by 5 minutes at 73 °C, 25 W. The N-terminal aminooxyacetic acid residue (AoAA) was incorporated manually. Briefly, N-Boc(aminooxy)acetic acid succinimidyl ester (2 molar excess) was dissolved in DMF (1 mL) and added to the peptidyl-resin. The mixture was shaken for 45 minutes, drained and washed with DMF (5 x 3 mL). The subsequent cleavage from the resin was achieved by incubating the resin in 10 mL of 94%TFA, 2.5% water, 2.5% DODT, 1% TIPS for 3 hours. Crude peptide was precipitated and triturated with cold diethyl ether, isolated (centrifugation), dissolved in 50% acetonitrile (aq) containing 0.1% TFA and lyophilized. The peptide was purified using a Dionex Ultimate 3000 reversed-phase HPLC system on a Phenomenex Gemini C18, 250 mm x 10 mm; 5µm column. The purified peptide showed a single peak with retention time of 15.75 minutes on LC-MS (Agilent Compact 1100 equipped with a 1100MSD mass spectrometer) using an Agilent SB-300 (C3, 3.0 mm x 150 mm; 3 □m) column. A linear gradient 5% to 65% of acetonitrile in water (both containing 0.1% formic acid) over 21 minutes was used. The mass signal at 539.2 m/z for the $[M+3H]^{3+}$ peak (calculated: 539.3) confirmed the identity of the desired product.

(2*S*,3*S*,4*R*)-2-(4-oxopentanoyl)-Val-Cit-(4-aminobenzyloxycarbonylamino)-1-(α-D-galactopyranosyloxy)-3-hydroxy-octadecan-4-yl hexacosanoate (17)



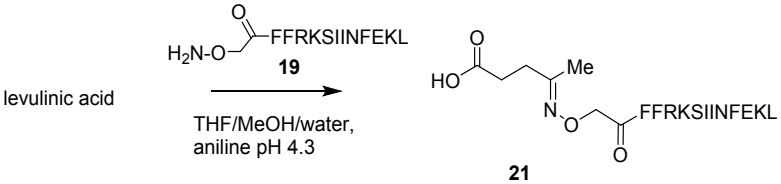
To a mixture of **1** (11 mg, 0.013 mmol) and pNP-carbonate **15** (13 mg, 0.020 mmol) in pyridine (0.17 mL) was added Et_3N (2.4 µL, 0.017 mmol) and the mixture was stirred at rt for 3 d. The reaction mixture was concentrated to dryness under high vacuum and the residue was triturated twice with water. The remaining solid was purified by column chromatography on silica gel ($MeOH/CHCl_3$ = 10:90 to 20:80), to afford the title compound **17** as a white solid (13 mg, 74%). ^1H NMR (500 MHz, 2:1 $CDCl_3/CD_3OD$) δ 0.87-0.90 (m, 6 H), 0.99-1.01 (m, 6 H), 1.22-1.40 (m, 68 H), 1.52-1.73 (m, 6 H), 1.76-1.84 (m, 1 H), 1.95-2.03 (m, 1 H), 2.08 (s, 3 H), 2.15-2.24 (m, 1 H), 2.31-2.41 (m, 2 H), 2.43-2.48 (m, 1 H), 2.57-2.62 (m, 1 H), 2.74-2.80 (m, 1 H), 2.89 (ddd, *J* = 5.3, 8.8, 18.7 Hz, 1 H), 3.12-3.24 (m, 2 H), 3.66-3.81 (m, 8 H), 3.85-3.88 (m, 2 H), 4.16 (d, *J* = 6.1 Hz, 1 H), 4.51 (dd, *J* = 4.6, 9.4 Hz, 1 H), 4.85 (d, *J* = 3.6 Hz, 1 H), 4.93-5.00 (m, 2 H), 5.09-5.16 (m, 1 H), 7.32 (d, *J* = 8.3 Hz, 2 H), 7.61 (d, *J* = 8.3 Hz, 2 H); ^{13}C NMR (126 MHz, 2:1 $CDCl_3/CD_3OD$) δ 14.2, 18.1, 19.4, 23.0, 25.4, 25.7, 26.8, 29.2, 29.56, 29.60, 29.69, 29.71, 29.74, 29.8, 29.90, 29.92, 29.95, 29.98, 30.01, 30.1, 30.4, 32.27, 32.29, 35.0, 38.8, 39.5, 52.6, 53.9, 60.1, 62.3, 66.8, 68.4, 69.4, 70.2, 70.7, 71.0, 72.3, 75.1, 100.4, 120.5, 129.0, 133.0, 138.3, 157.1, 161.0, 171.1, 172.9, 174.7, 175.0, 210.0; HRMS-ESI $[M+Na]^+$ calcd for $C_{74}H_{132}N_6NaO_{16}$: 1383.9598; found 1383.9576.

4-Pentynoyl-FFRKSIINFEKL (20)



Peptide **20** was synthesised by Fmoc solid phase peptide synthesis on an aminomethyl polystyrene resin (loading 0.87 mmol/g) from Rapp Polymer (Tubingen, Germany)). Where necessary Nα-amino acids with the following protecting groups were used: Arg(Pbf), Lys(tBu), Ser(tBu), Asn(Trt) and the peptide was synthesised using a Biotage Alstra microwave peptide synthesiser (John Morris, Sydney, Australia). The synthesis was conducted on a 0.1 mmol scale using reaction cycles consisting of Nα-Fmoc deprotection with 20% piperidine in DMF 1 x 3 mins then 1 x 10 mins at room and couplings (5 minutes, 73 °C, 25 W) employing a 5-fold molar excess of the protected amino acid in DMF activated by a 4.5 molar excess of HCTU in the presence of an 10 molar excess of DIPEA. Nα-Fmoc-Arg(Pbf) was coupled for 25 minutes at room temperature followed by 5 minutes at 73 °C, 25 W. The N-terminal 4-pentynoic acid was incorporated manually. Briefly, 4-pentynoic acid (10 molar excess), HCTU (9.5 molar excess) were dissolved in DMF (3 mL), DIPEA (20 molar excess) was added and the solution added to the peptidyl-resin. The mixture was shaken for 1 hour, drained and washed with DMF (5 x 3 mL), MeOH (3 x 10 mL) and dried. The subsequent cleavage from the resin was achieved by incubating the resin in 10 mL of 95%TFA, 2.5% water, 2.5% TIPS for 3 hours. Crude peptide was precipitated and triturated with cold diethyl ether, isolated (centrifugation), dissolved in 50% acetonitrile (aq) containing 0.1% TFA and lyophilized to afford 145 mg of peptide. The peptide was purified in 2 x 30 mg batches using a Dionex Ultimate 3000 reversed-phase HPLC system on a Phenomenex Gemini C18, 250 mm x 10 mm; 5µm column. The purified peptide (24.84 mg) showed a single peak with retention time of 16.7 minutes on LC-MS (Agilent Compact 1100 equipped with a 1100MSD mass spectrometer) using an Agilent SB-300 (C3, 3.0 mm x 150 mm; 3 □m) column. A linear gradient 5% to 65% of acetonitrile in water (both containing 0.1% formic acid) over 21 minutes was used. The mass signal at 541.5 m/z for the $[M+3H]^{3+}$ peak (calculated: 541.6) and 811.5 m/z for the $[M+2H]^{2+}$ peak (calculated: 811.9) confirmed the identity of the desired product.

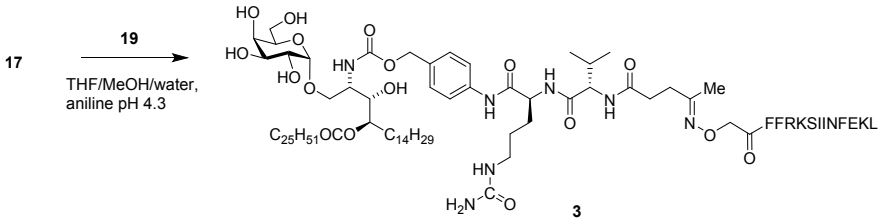
4-((2-(FFRKSIINFEKL)-2-oxoethoxy)imino)pentanoic acid (21)



To a stirred suspension of peptide 2-(aminooxy)acetyl-FFRKSIINFEKL (6.0 mg, 3.72 mmol) in THF/MeOH (2:1, 600 µL) was added an aqueous mixture of water/aniline/TFA (200:6:4, 300 µL, pH 4.3). Once dissolved, a solution of levulinic acid (100 mg, 0.86 mmol) dissolved in MeOH (200 µL) was added and the reaction mixture was stirred at 25 °C for 48 h. The solvent was removed and the crude product was purified by preparative HPLC (Phenomenex Luna C18(1), 5 µm, 250 x 10 mm, 40 °C, 1.4 mL/min; Mobile phase A = 100:0.1 water/ TFA; Mobile phase B = 100:0.1 MeOH/TFA; 0-10 min: 50-

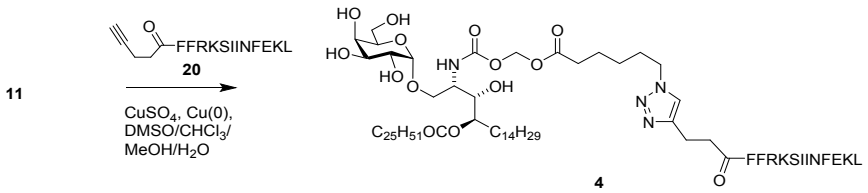
100% B; 10-15 min: 100% B; 15-16 min: 100-50% B; 16-20 min: 50% B) to give the title compound **21** (3.9 mg, 62%, 96% pure by HPLC). ^{1}H NMR (500 MHz, d6-DMSO) δ 0.70-0.88 (m, 18H), 0.99-1.11 (m, 2H), 1.24-1.43 (m, 7H), 1.44-1.60 (m, 12H), 1.60-1.577 (m, 8H), 1.79 (s, 2H), 1.91 (s, 1H), 2.17-2.30 (m, 2H), 2.31-2.40 m, 3H), 2.67-2.96 (m, 9H), 2.98-3.16 (m, 4H), 3.54-3.62 (m, 4H), 4.11-4.62 (m, 15H), 5.00 (br s, 1H), 6.92 (s, 1H), 7.11-7.29 (m, 17H), 7.36 (d, *J* = 7.9 Hz, 1H), 7.41 (s, 1H), 7.45-7.53 (m, 1H), 7.57-7.87 (m, 8H), 7.91-8.21 (m, 8H); HRMS (ESI): *m/z* calcd for $C_{82}H_{126}N_{19}O_{21}$ $[M+H]^+$ 1712.9376, found 1712.9366

(2*S*,3*S*,4*R*)-2-((4-(2-((FFRKSIINFEKL)-2-oxoethoxy)imino)pentanoyl)-Val-Cit-(4-aminobenzyloxycarbonylamino)-1-(α-D-galactopyranosyloxy)-3-hydroxy-octadecan-4-yl hexacosanoate (3)



Aniline buffer (300 mM, pH 4.3) was prepared by mixing aniline (56 mg) and TFA (58 mg) in water and diluting to 2.0 mL. A mixture of peptide **19** (2.1 mg, 1.0 μmol) and ketone **17** (1.4 mg, 1.3 μmol) was stirred in THF/MeOH/aniline buffer (4:2:3, 450 μL) at 25 °C for 48 h. The mixture was diluted with DMSO and purified by preparative HPLC (Phenomenex Luna C18(1), 5 μm, 250 x 10 mm, 35 °C, 2.5 mL/min; Mobile phase A = 100:0.05 water/TFA; Mobile phase B = 100:0.05 MeOH/TFA; 0-6 min: 80-100% B; 6-12 min: 100% B; 12-12.5 min: 100-80% B; 12.5-15 min: 80% B) to give the title compound **3** (1.0 mg, 34%, 98.8% pure by HPLC); HRMS-ESI *m/z* calcd for $C_{151}H_{251}N_{25}O_{34}$ $[M+2H]^{2+}$ 1479.4340, found 1479.4355.

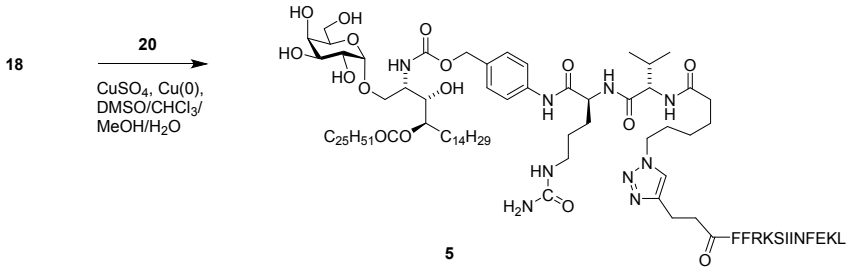
(2*S*,3*S*,4*R*)-2-((((6-(4-(3-(FFRKSIINFEKL)-3-oxopropyl)-1*H*-1,2,3-triazol-1-yl)hexanoyl)oxy)methoxycarbonyl)amino)-1-(α-D-galactopyranosyloxy)-3-hydroxy-octadecan-4-yl hexacosanoate (4)



To a stirred solution of peptide 4-pentynoyl-FFRKSIINFEKL (**20**) (4.5 mg, 2.80 μmol) and **11** (2.30 mg, 2.15 μmol) in DMSO (600 μL) and MeOH (280 μL) was added TBTA (0.33 mg, 0.6 μmol) in $CHCl_3$ (280 μL) followed by an aqueous solution of 2.5 mM $CuSO_4$ (100 μL). A small piece of copper foil (5 mm x 2 mm) was added and the reaction mixture was stirred at rt for 18 h. After evaporation of the volatiles

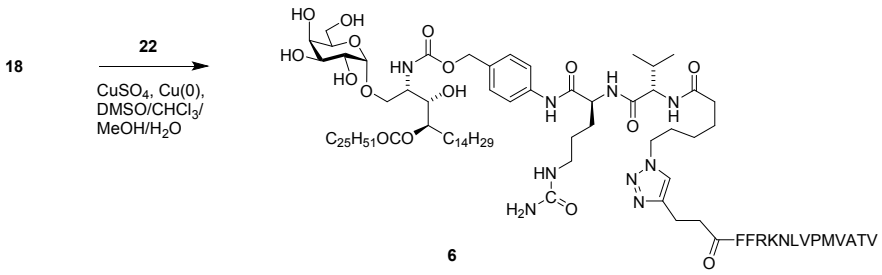
under an Ar stream, the product was precipitated by addition of aq 0.05 M EDTA and formic acid (pH 4, 10 mL) and separated by centrifugation. The pellet was spun again with further EDTA (10 mL) and water (2 x 10 mL), and dried under vacuum. The crude product was purified by preparative HPLC (Phenomenex Luna C18(1), 5 µm, 250 x 10 mm, 40 °C, 2.1 mL/min; Mobile phase A = 100:0.05 water/TFA; Mobile phase B = 100:0.05 MeOH/TFA; 0-7 min: 80-100% B; 7-14 min: 100% B; 14-15 min: 100-80% B; 15-20 min: 80% B) to give the title compound **4** (2.55 mg, 44%, 98.7% pure by HPLC); HRMS-ESI *m/z* calcd for $C_{138}H_{232}N_{22}O_{31}$ $[M+2H]^{2+}$ 1346.8627, found 1346.8605.

(2*S*,3*S*,4*R*)-2-(6-(4-(3-(FFRKSIINFEKL)-3-oxopropyl)-1*H*-1,2,3-triazol-1-yl)hexanoyl)-(*N*-Val-Cit-4-aminobenzyloxycarbonylamino)-1-(α-D-galactopyranosyloxy)-3-hydroxy-octadecan-4-yl hexacosanoate (5)



To a stirred solution of peptide 4-pentynoyl-FFRKSIINFEKL (**20**) (9.5 mg, 5.80 µmol), **18** (6.0 mg, 4.2 µmol) and TBTA (0.75 mg, 1.4 µmol) in DMSO (280 µL) was added $CHCl_3$ (280 µL) and MeOH (280 µL) followed by an aqueous solution of 2.5 mM $CuSO_4$ (107 µL). A small piece of copper foil (5 mm x 2 mm) was added and the reaction mixture was stirred at 20 °C for 48 h. After evaporation of the volatiles under an Ar stream, the product was precipitated by addition of aq 0.05 M EDTA (pH 7.7, 10 mL) and separated by centrifugation. The pellet was spun again with further EDTA (10 mL) and water (2 x 10 mL), and dried under vacuum. The crude product was purified by preparative HPLC (Phenomenex Luna C18(2), 5 µm, 250 x 30 mm, 30 °C, 40 mL/min; Mobile phase A = 100:0.1 water/ TFA; Mobile phase B = 100:0.1 MeOH/TFA; 0-15 min: 50-100% B; 15-23 min: 100% B; 23-25 min: 100-50% B; 25-26 min: 50% B) to give the title compound **5** (6.0 mg, 46.4%, 97.5% pure by HPLC); HRMS-ESI *m/z* calcd for $C_{155}H_{257}N_{27}O_{33}$ $[M+2H]^{2+}$ 1512.4631, found 1512.4618.

(2*S*,3*S*,4*R*)-2-(6-(4-(3-(FFRKNLVPMVATV)-3-oxypropyl)-1*H*-1,2,3-triazol-1-yl)hexanoyl)-(*N*-Val-Cit-4-aminobenzyloxycarbonylamino)-1-(α-D-galactopyranosyloxy)-3-hydroxy-octadecan-4-yl hexacosanoate (6)



To a stirred solution of peptide 4-pentynoyl-FFRKNLVPMVATV (**22**) (2.0 mg, 1.25 µmol), **18** (1.0 mg, 0.71 µmol) and TBTA (0.29 mg, 0.55 µmol) in DMSO (93 µL) was added $CHCl_3$ (93 µL) and MeOH (93 µL) followed by an aqueous solution of 2.5 mM $CuSO_4$ (31 µL). A small piece of copper foil (5 mm x 2 mm) was added and the reaction mixture was stirred at 20 °C for 15 h. After evaporation of the volatiles under an Ar stream, the product was precipitated by addition of aq 0.025 M EDTA (pH 7.7, 10 mL) and separated by centrifugation. The pellet was spun again with further EDTA (10 mL) and water (2 x 10 mL), and dried under vacuum. The crude product was purified by preparative HPLC (Phenomenex Luna C18(2), 5 µm, 250 x 30 mm, 30 °C, 40 ml/min; Mobile phase A = 100:0.1 water/TFA; Mobile phase B = 100:0.1 MeOH/TFA; 0-15 min: 50-100% B; 15-23 min: 100% B; 23-25 min: 100-50% B; 25-26 min: 50% B) to give the title compound **6** (1.65 mg, 77%, 94.1% pure by HPLC); HRMS-ESI *m/z* calcd for $C_{152}H_{257}N_{27}O_{32}S$ $[M+2H]^{2+}$ 1502.4517, found 1502.4492.

HPLC-ESI-MSMS Quantification of α-GalCer in aqueous samples of 2-5

Stock samples of compounds **2-5** were solubilized at 0.5 mg/mL in sucrose, L-histidine and Tween 20 as previously described for the solubilisation of α-GalCer.[4] he stock samples were further diluted to 10 ug/mL of **2-5** and 0.2 ug/mL of d_4-α-GalCer[5] in 50 mM aqueous buffers at four pH levels (pH 3.0 ammonium formate, pH 5.0 ammonium acetate, pH 7.4 *N*-ethylmorpholonium acetate, pH 9.0 ammonium acetate). Quantification of the amount of α-GalCer in compounds **2** - **5** was made by HPLC-ESI-MSMS analysis using a Waters 2795 HPLC and a Waters Q-TOF Premier™ Tandem Mass Spectrometer. The chromatography used a Phenomenex Kinetex C18 2.6 mm 3.0 x 50 mm column eluting with isocratic methanol containing 10 mM ammonium formate + 0.5% formic acid at a flow rate of 0.2 mL/min. α-GalCer was monitored by selective reactant monitoring of *m/z* 858.7 to 696.7 and quantified by comparison to d_4-αGalCer as an internal standard (m/z 862.7 to 700.7). Samples were incubated at 20 °C and sampled periodically for α-GalCer quantification over eight days with the eight-day level of aGalCer shown in the Table 1.

	pH 3.0	**pH 5.0**	**pH 7.4**	**pH 9.0**
2	0.2 %	0.5 %	0.9 %	2.6 %
3	<0.05 %	<0.05 %	<0.05 %	<0.05 %
4	<0.05 %	<0.05 %	0.2 %	0.9 %
5	<0.05 %	<0.05 %	<0.05 %	<0.05 %

Table 1 Level of α-GalCer after eight days at pH 3, 5, 7.4 and 9

Mice and ethical approval. C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA), CD1d$^{-/-}$ mice[6], and and OT-I mice, which express a transgenic TCR specific for the H-2K^{b}-binding peptide of OVA (OVA_{257})[7], were bred at the Malaghan Institute of Medical Research, and used at 6-12 weeks of age. All experiments were approved by the Victoria University Animal Ethics Committee and performed according to institutional guidelines.

Solubilization and administration of compounds for biological studies. Solubilization of α-GalCer, and **2**-**6** was achieved by freeze-drying the samples in the presence of sucrose, L-histidine and Tween 20 as previously described for the solubilisation of α-GalCer.[4] All solubilized compounds were diluted in water for i.v. administration. Equivalent molar doses were used in all groups in a given experiment.

Assessment of dendritic cell activation *in vivo*. The experimental compounds were administered i.v. into groups of C57BL/6 mice (*n* = 3), and the spleens were removed 20 h later for analysis. The spleens were teased through a cell strainer and the red blood cells (RBCs) lysed with RBC lysis buffer, and then the remaining cells were labeled with antibodies for CD11c (HL3, BD Pharmingen), and CD86 (GL1, eBioscience) and propidium iodide (BD Pharmingen) and analysed by flow cytometry. All antibody staining steps were performed on ice. Nonspecific FcR-mediated Ab staining was blocked by incubation for 5 min with anti-CD16/32 antibody (24G2, prepared in-house from hybridoma supernatant). Flow cytometry was performed on a BD Biosciences FACSCalibur or BD Biosciences LSRII SORP with data analysis using FlowJo software (Tree Star, Inc., OR, USA).

***In vivo* cytotoxicity assay.** As targets, four syngeneic splenocytes populations were prepared; cells loaded with 50 nM OVA_{257} peptide and labeled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), cells loaded with 5 nM OVA_{257} peptide and labeled with 0.5 μM CFSE, cells loaded with 0.5 nM OVA_{257} peptide and labeled with 0.1 μM CFSE, and cells without peptide labeled with 10 μM 4-((chloromethyl)benzoylamino)tetramethylrhodamine (Life Technologies). A mixture of the four populations was injected i.v into immunised mice, and specific lysis of the peptide-loaded targets was monitored by flow cytometry analysis of PBL. Mean percentage of survival of peptide-pulsed targets was calculated relative to that of the control population, and cytotoxic activity was expressed as percent specific lysis (100 - mean percentage of survival of peptide-pulsed targets). Mean percent specific lysis per treatment group (*n* = 5 mice) ± SEM is presented.

Assessment of peptide presentation *in vitro*. Splenocytes were prepared from OT-I mice and plated at 2 x 10^5 cells per well in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% fetal bovine serum (SAFC Bioscience, Auckland, New Zealand), 100 U/mL penicillin, 100 g/mL streptomycin, and 50 μM 2-mercaptoethanol (all from Invitrogen). The indicated doses of the experimental compounds were added to the wells in triplicate, and the cultures were incubated for 72 h at 37 °C. Proliferation was assessed as uptake of ^{3}H thymidine over the last 8 h of culture. All cultures were harvested on a Tomtec 96-well automated cell harvester (Orange, CT) and counted on a Wallac 1450 Microbeta Plus counter (Turku, Finland).

Assessment of expression of H-2K^b/OVA$_{257}$ complexes on cell surface. Cells from the murine dendritic cell line DC2114[8] were plated at 3 x 10^5 cells in IMDM and incubated with the indicated doses of the experimental compounds for 6, 12 or 24 h before flow cytometry was performed. Nonspecific FcR-mediated Ab staining was blocked by incubation for 5 min with anti-CD16/32 antibody, and then cells were stained with monoclonal antibody for H-2K^b/OVA$_{257}$ complexes (25-D1.16, Biolegend). Samples were analysed using a BD Biosciences LSR-II flow cytometer.

Therapeutic Tumour model. The melanoma cell-line B16.OVA, which express OVA protein,[9] was cultured in IMDM supplemented with 5 % FBS, 2 mM glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol (all Invitrogen), strained through a 70 μm filter, and resuspended in PBS for s.c. injection. Groups of naïve C57BL/6 mice (n = 7) were challenged with 5 × 10^5 cells in the flank and then on day 5, when tumors were palpable, were subjected to one round of i.v. therapy as indicated in text, or PBS. Tumor growth was monitored every 2-3 days, with tumor size calculated as the product of the two bisecting diameters. Measurements were stopped for each group when the first mouse developed a tumor in excess of 200 mm^2.

Analysis of activation of human NKT cells *in vitro*. Peripheral blood mononuclear cells (PBMCs) from an HLA-A*02 negative healthy human donor were cultured with 500 ng/ml (583 nM) α-GalCer, or molar equivalent of vaccine **6**, at 3 x 10^5 cells per well for 72 hours in presence of 50 μg/ml anti-CD1d (clone 51.1; BioLegend, CA, USA) or matched isotype control. Induction of IFN-γ release was assessed by human IFN-γ ELISpot kit (Mabtech, Nacka Strand, Sweden). Activation status of NKT cells was assessed in similar cultures after 18 hours by flow cytometry using anti-iNKT (6B11, BD Pharmingen), anti-CD3 (UCHT1; BioLegend), and anti-CD137 (4B4-1; BioLegend).

Stimulation of human CMV-specific CD8$^+$ T cells *in vitro*. PBMCs from HLA-A*02 positive CMV-

seropositive donors were cultured at 3 x 10^5 cells per well for 7 days with α-GalCer, NLV peptide, admixed peptide and α-GalCer or vaccine **6**, at equimolar concentrations. Frequency and activation status of peptide-specific $CD8^+$ T cells as a percentage of all T cells was determined by flow cytometry using fluorescent-labelled NLV-loaded HLA-A*02 dextramer (Immudex, Copenhagen, Denmark), anti-CD8 (RPA-T8; BioLegend) and anti-CD3 (OKT3; BioLegend) and anti-CD137 (4B4-1; BioLegend).

Statistical analysis. Data presented in graphs were analysed by 1-way ANOVA with multiple comparisons by Tukey test using Prism software (GraphPad Software Inc., La Jolla, CA). *P<0.05, *P<0.01, ***P<0.001.

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