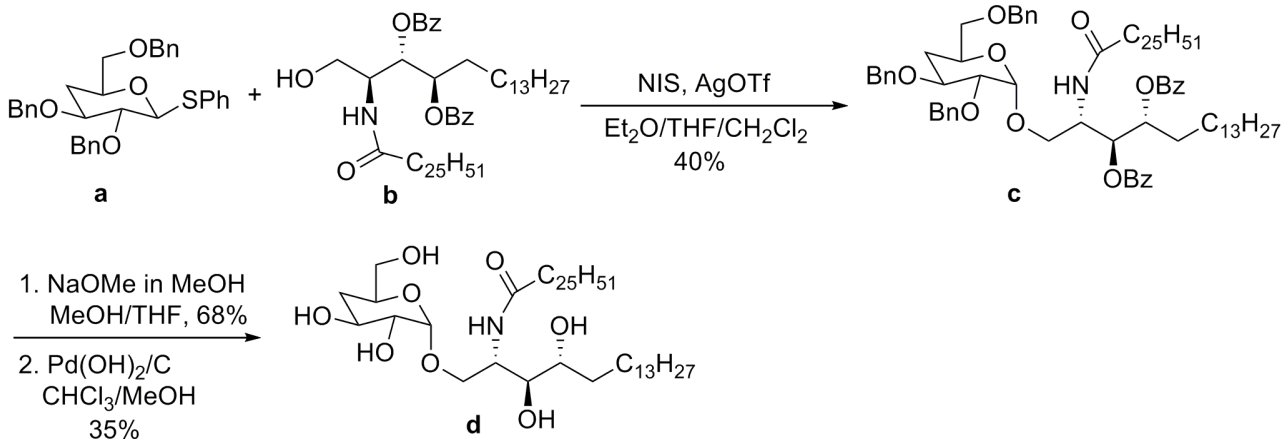
Supplementary information

Supplementary experimental procedures

Glycolipid synthesis

4′-Deoxy-α-GalCer **d** was prepared by the coupling of known armed sugar donor[1] **a** and disarmed acceptor[2] **b**, followed by a two stage deprotection of glycosylceramide **c**.



1. “Synthesis and Evaluation of 3”- and 4”-Deoxy and -Fluoro Analogs of the Immunostimulatory Glycolipid, KRN7000,” Raju, R.; Castillo, B. F.; Richardson, S. K.; Thakur, M.; Severins, R.; Kronenberg, M.; Howell, A. R. *Bioorg. Med. Chem. Lett*. **2009**, *19*, 4122-4125.

2. "Synthesis and Biological Evaluation of α-Galactosylceramide (KRN7000) and Isoglobotrihexosylceramide (iGb3)," Xia, C.; Yao, Q.; Schumann, J.; Rossy, E.; Chen, W.; Zhu, L.; Zhang, W.; Libero, G. D.; Wang, P. G. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2195-2199.

Supplementary information – crystallography

Data were processed with programs from the CCP4 suite (CCP4, 1994). Crystals of the different ternary complexes diffracted to a range of 2.7 Å - 3.0 Å, and belong to the space group $P2_12_12_1$. Crystal structures of the different NKT TCR-CD1d-Ag complexes were solved using Phaser using the Vβ8.2 NKT TCR-CD1d-α-GalCer complex (Protein Data Bank ID code 3HE6) minus α-GalCer as the search model, and subsequently refined using Refmac. To prevent model bias, FreeR of the NKT TCR-CD1d-α-GalCer structure was used in the experimental intensities scaling process in Scala as well as the implementation of the simulated annealing protocol in Phenix (Zwart et al., 2008). Glycolipid libraries were generated via the program Sketcher, and the glycolipid models were subsequently built into the work models. Restrained refinement followed by the inclusion of translation libration screw parameters at the later stage interspersed with rounds of model building with Coot (Emsley and Cowtan, 2004) were used to improve the work model as monitored by the R_{free} values. Programs within the CCP4 suite were utilized to assess the quality of the structures. For data collection and refinement statistics, see Supplementary Table 2. All molecular graphics illustrations were generated using PyMol (DeLano, 2002). The residues that could not be modelled in the NKT TCR-CD1d-OCH were: CD1d: residues 1-5, and 91-92; β_2m: residue 1; TCR α-chain: residues 185, and 208-210; and TCR β-chain: residues 1-2, and 97-102 (CDR3β). The residues that could not be modelled in the NKT TCR-CD1d-C20:2 complex were: CD1d: residues 1-6, 108, and 301-302; TCR α chain: residues 185 and 208-210; and TCR β chain: residues 1-3 and 97-102 (CDR3β); C20:2: sphingosine chain: C18; and acyl chain: C7-C18.The residues that could not be modelled in the NKT TCR-CD1d-α-GlcCer complex were: CD1d: residues 1-6, 89, 108-109, 241 and 301-302; β_2m: residue 1; TCR α-chain: residues 99 (CDR3α), 185, and 208-210; and TCR β chain: residues 1-2, 97-101 (CDR3β) and 247; -α-GlcCer: sphingosine chain: C9-C18; and acyl chain: C4-C20. The residues that could not be modelled in the NKT TCR-CD1d-3′,4′′-deoxy-α-GalCer were: CD1d: residues 1-5, 108 and 301-302; TCR α chain: residues 185-186, and 208-210; and TCR β chain: residues 1-2, and 97-100 (CDR3β); 3′,4′′-deoxy-α-GalCer: sphingosine chain: C12-18. The residues that could not be modelled in the NKT TCR-CD1d-4′,4′′deoxy-α-GalCer were: CD1d: residues 1-6, 90, 108, and 301-302; TCR α chain: 185, and 208-210; and TCR β chain: 1-2, and 98-101 (CDR3β); 4′,4′′-deoxy-α-GalCer: acyl chain: C4-5.

Supplementary Figure Legends

Supplementary Fig. 1 Serum cytokine analysis. Male C57BL/6 mice (n=3) were injected i.p. with 1μg of glycolipid or the equivalent volume of vehicle buffer in 100μl PBS and bleed at 2hr and 20hr time-points. Serum was isolated via centrifugation and IFN-γ and IL-4 levels were quantified by Cytometric Bead Array (BD Biosciences). Error bars represent SEM.

Supplementary Fig. 2. Electron density for the AGL ligands, as omit maps and final 2Fo-Fc maps that highlights that the positioning of the various head groups are unambiguous

Supplementary Table 1. Surface Plasmon Resonance measurement of the mouse NKT TCR to CD1d-α-GalCer and analogues

CD1d-glycolipids	Mouse NKT TCR Vβ8.2					
	K_{Deq} (nM)	k_a (x 10^5 $M^{-1}s^{-1}$)	k_d (s^{-1})	$t_{1/2}$ (sec)	K_D calc (nM)	Chi^2
αGalCer	59.6	5.84	0.03	19.69	54.7	3.72
OCH	287	1.87	0.04	14.35	234	3.31
α-GalCer (C20:2)	68.4	6.31	0.04	15.48	64.4	4.07
α-GlcCer (C20:2)	684	4.69	0.30	2.08	645	2.22
3′,4′′-deoxy-α-GalCer	174	5.99	0.11	6.00	175	2.67
4′,4′′-deoxy-α-GalCer	423	3.88	0.24	2.62	617	6.37
4′-deoxy-α-GalCer	525	4.01	0.22	2.83	556	9.31
α-GalCer (C24)	49.3	6.77	0.03	21.14	44.1	4.24
α-GlcCer (C24)	554	2.40	0.20	3.47	814	6.44

K_{Deq} derived by equilibrium fit option
K_D calc derived by kinetic fit
$t_{1/2} = 0.693/k_d$

Supplementary Table 2 Data collection and refinement statistics

	NKT TCR-mCD1d-C20:2	NKT TCR-mCD1d-α-GlcCer	NKT TCR-mCD1d-3′,4″-deoxy-α-GalCer	NKT TCR-mCD1d-4′,4″-deoxy-α-GalCer	NKT TCR-mCD1d-OCH
Data Collection					
Resolution limits (Å)	50-2.9 (3.06-2.90)	50-3.0 (3.16-3.00)	50-3.0 (3.16-3.00)	50-2.8 (2.95-2.80)	50-2.7 (2.85-2.70)
Space Group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions	*a*=58.95, *b*=87.05, *c*=234.86; α=β=γ=90.00°	*a*=59.17, *b*=85.77, *c*=237.81; α=β=γ=90.00°	*a*=58.99, *b*=86.73, *c*=235.20; α=β=γ=90.00°	*a*=58.58, *b*=86.26, *c*=236.10; α=β=γ=90.00°	*a*=59.04, *b*=87.31, *c*=235.65; α=β=γ=90.00°
Total $N^{o.}$ observations	425891	245371	234455	200512	118283
$N^{o.}$ unique observations	27541	23592	24812	28434	32741
Multiplicity	15.5 (15.8)	10.4 (10.5)	9.4 (9.5)	7.1 (6.9)	3.6 (3.6)
Data Completeness	100 (100)	94.5 (95.9)	100 (100)	93.3 (91.0)	95.9 (97.4)
I/σ	9.7 (2.5)	9.4 (2.0)	9.3 (2.0)	9.7 (2.0)	9.3 (2.1)
$^1R_{p.i.m}$ (%)	9.6 (40.1)	6.9 (34.0)	6.8 (36.6)	7.4 (34.3)	5.2 (33.1)
Mosaicity	0.87	0.49	0.53	0.68	0.45
Refinement statistics					
$^2R_{factor}$ (%)	22.5	23.2	21.3	22.0	21.4
$^3R_{free}$ (%)	27.7	30.0	27.7	27.9	25.7
Non hydrogen atoms					
- protein	6337	6038	6318	6434	6475
- lipid(s)	54	54	58	58	61
- water	35	22	18	55	68
- other	75	63	72	70	102
Ramachandran plot					
- Most favoured (%)	90.4	89.0	90.4	90.1	91.6
- Allowed region (%)	9.6	11.0	9.6	9.9	8.4
B-factors (Å^2)					
- Average main chain	58.2	73.6	73.6	56.4	57.2
- Average side chain	58.2	72.6	73.7	56.5	57.5
- lipid(s)	49.9	75.7	61.3	59.3	50.4
- water	51.4	63.1	64.5	41.0	50.8
rmsd bonds (Å)	0.006	0.006	0.006	0.006	0.006
rmsd angles (°)	0.941	0.992	0.920	0.897	0.943

$^1R_{p.i.m} = \Sigma_{hkl} [1/(N-1)]^{1/2} \Sigma_i | I_{hkl,\,i} - \langle I_{hkl} \rangle | / \Sigma_{hkl} \langle I_{hkl} \rangle$

$^2R_{factor} = \Sigma_{hkl} | | F_o | - | F_c | | / \Sigma_{hkl} | F_o |$ for all data except ≈ 5% which were used for $^3R_{free}$ calculation

Values in parentheses refer to the highest resolution bin

Supplementary Table 3. Contacts Table between NKT TCR and CD1d-analogue complexes

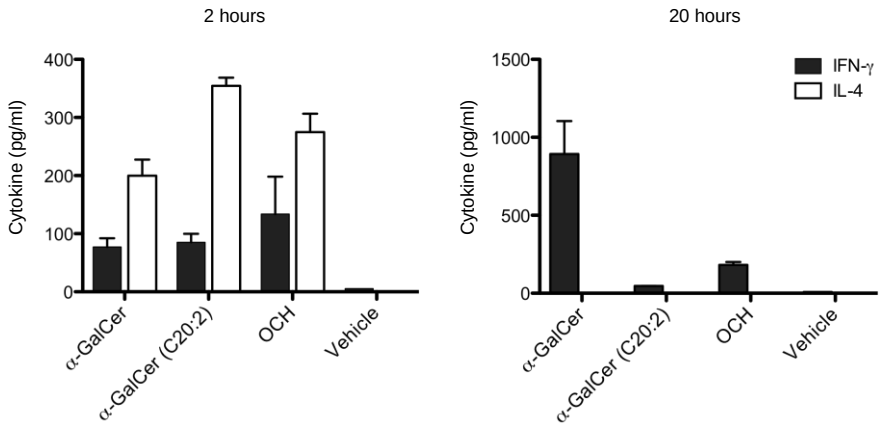
CDR	**NKT Vβ 8.2**	**NKT TCR-CD1d-α-GalCer**		**NKT TCR-CD1d-C20:2**	**NKT TCR-CD1d-α-GlcCer**	**NKT TCR-CD1d-3′,4′′-deoxy-α-GalCer**	**NKT TCR-CD1d-4′,4′′-deoxy-α-GalCer**	**NKT TCR-CD1d-OCH**
		CD1d	**Bond**	**CD1d**	**CD1d**	**CD1d**	**CD1d**	**CD1d**
CDR1α	Thr27					***Val72*** (VDW)		***Val72*** (VDW)
	Pro28			**Val72** (VDW)	***Ser76*** (VDW)	Val72, ***Ser76*** (VDW)	Val72, ***Ser76*** (VDW)	Val72, ***Ser76*** (VDW)
CDR3α	$Asp94^{O}\delta_1$	$Arg79^{N}\eta_1$, $Arg79^{N}\eta_2$	Salt bridge	$Arg79^{N}\eta_1$, $Arg79^{N}\eta_2$	Same as C20:2	Same as C20:2	Same as C20:2	Same as C20:2
	$Asp94^{O}\delta_2$	$Arg79^{N}\eta_1$, $Arg79^{N}\eta_2$	Salt bridge	$Arg79^{N}\eta_1$, $Arg79^{N}\eta_2$	Same as C20:2	Same as C20:2	Same as C20:2	Same as C20:2
	Asp94	Arg79	VDW	Arg79	Same as C20:2	Same as C20:2	Same as C20:2	Same as C20:2
	$Arg95^{N}\varepsilon$	$Asp80^{O}\delta_1$, $Asp80^{O}\delta_2$	Salt bridge	$Asp80^{O}\delta_1$, $Asp80^{O}\delta_2$	Same as C20:2	*Not seen	Same as C20:2	Same as C20:2
	$Arg95^{N}\eta_1$	$Asp80^{O}\delta_1$	Salt bridge	$Asp80^{O}\delta_1$	Same as C20:2	*Not seen	Same as C20:2	Same as C20:2
		$Ser76^{O}\gamma$	H bond	**$Arg79^{N}\varepsilon$**	Same as C20:2	***$Ser76^{O}$***	-	***$Ser76^{O}$***
	$Arg95^{N}\eta_2$	-	-	-	***$Asp80^{O}\delta_1$*** (Salt bridge)	-	***$Arg79^{N}\eta_2$*** (H bond)	-
	Arg95	Ser76, Arg79, Asp80	VDW	Ser76, Arg79, Asp80	Same as C20:2	Ser76, Arg79	Same as C20:2	Same as C20:2
	$Gly96^{N}$	$Asp153^{O}\delta_2$	H bond	-	-	-	-	-
	Gly96	Ala152, Asp153	VDW	Ala152, Asp153	Same as C20:2	Same as C20:2	Same as C20:2	Same as C20:2
	Ser97	Val149	VDW	Val149	Same as C20:2	Val149, ***Asp153***	-	Same as C20:2
	$Leu99^{O}$	$Arg79^{N}\eta_2$	H bond	$Arg79^{N}\eta_2$	*Not seen	Same as C20:2	-	-
	Leu99	Arg79, Val149	VDW	Val149, **Asp80**, **Glu83**, **Leu84**	*Not seen	Val149, Glu83	***Arg79***, Val149, Asp80, Leu84	***Arg79***, Val149, Asp80, Leu84
	Gly100	Arg79	VDW	-	Arg79	Arg79	-	-
	$Arg103^{N}\eta_1$	$Glu83^{O}\varepsilon_1$, $Glu83^{O}\varepsilon_2$	Salt bridge	$Glu83^{O}\varepsilon_1$, $Glu83^{O}\varepsilon_2$	*Not seen	Same as C20:2	Same as C20:2	Same as C20:2
	$Arg103^{N}\eta_2$	-	-	**$Glu83^{O}\varepsilon_1$**, (Salt bridge)	-	-	-	-
	Arg103	Arg79, Glu83	VDW	Arg79, Glu83	Arg79	Same as C20:2	Same as C20:2	Arg79

CDR2β	Tyr48$^{O}\eta$	Glu83$^{O}\varepsilon_1$, Glu83$^{O}\varepsilon_2$, Lys86$^{N}\zeta$	H bond	Glu83$^{O}\varepsilon_1$	Same as C20:2	Same as C20:2	Same as C20:2	Same as C20:2
	Tyr48	Glu83, Lys86	VDW	Glu83, Lys86	Glu83	Same as C20:2	Same as C20:2	Same as C20:2
	Tyr50$^{O}\eta$	Glu83$^{O}\varepsilon_1$	H bond	Glu83$^{O}\varepsilon_1$	Same as C20:2	Same as C20:2	Same as C20:2	Same as C20:2
	Tyr50	Glu83, Met87	VDW	Glu83, Met87	Same as C20:2	Same as C20:2	Same as C20:2	Same as C20:2
	Glu56$^{O}\varepsilon_1$	Lys86$^{N}\zeta$	Salt bridge	-	*Not seen	-	-	***Lys86***$^{N}\zeta$
	Glu56	Lys86	VDW	Lys86	*Not seen	Same as C20:2	Same as C20:2	Same as C20:2

CDR	**NKT Vβ 8.2**	**α-GalCer**	**Bond**	**C20:2**	**α-GlcCer**	**4′,4″-deoxy-α-GalCer**	**4′-deoxy-α-GalCer**	**OCH**
CDR1α	Pro28	6′-OH^{G}, 5′-O^{G}, C-1^{G}	VDW	6′-OH^{G}, 5′-O^{G}, C-1^{G}	5′-O^{G}, C-1^{G}, **1′-O^{G}**, C-1^{S}	6′-OH^{G}, 5′-O^{G}	6′-OH^{G}, 5′-O^{G}	**C-6^{G}**, 5′-O^{G}, 6′-OH^{G}, C-1^{G}
	Asn30$^{N}\delta_2$	3′-OH^{G}, 4′-OH^{G}	H bond	3′-OH^{G}, 4′-OH^{G}	3′-OH^{G}	4′-OH^{G}	3′-OH^{G}	3′-OH^{G}, 4′-OH^{G}
	Asn30	C-2^{G}, C-3^{G}, C-4^{G}, 3′-OH^{G}, 4′-OH^{G}	VDW	C-3^{G}, 3′-OH^{G}, 4′-OH^{G}	C-2^{G}, C-3^{G}	C-2^{G}, C-3^{G}, C4^{G}, 4′-OH^{G}	-	C-2^{G}, C-3^{G}, C-4^{G}, 3′-OH^{G}, 4′-OH^{G}
CDR3α	Asp94^{O}	C-1^{G}	VDW	C-1^{G}, **C-2^{G}**	-	-	-	C-1^{G}
	Arg95$^{N}\varepsilon$	-	-	-	-	-	-	**3′-OH^{S}** (H bond)
	Arg95	2′-OH^{G}, C-2^{G}, 3′-OH^{S}	VDW	2′-OH^{G}, C-2^{G}, 3′-OH^{S}	2′-OH^{G}, **1′-O^{G}**, 3′-OH^{S}	2′-OH^{G}, **C-1^{G}**, C-2^{G}, 3′-OH^{S}	2′-OH^{G}, **C-1^{G}**, **1′-O^{G}**, **C-1^{S}**, 3′-OH^{S}	2′-OH^{G}, C-2^{G}, 3′-OH^{S}
	Gly96^{N}	2′-OH^{G}	H bond	2′-OH^{G}	-	2′-OH^{G}	-	2′-OH^{G}
	Gly96	C-2^{G}, 3′-OH^{G}	VDW	C-2^{G}, 3′-OH^{G}	**2′-OH^{G}**	C-2^{G}	**2′-OH^{G}**	C-2^{G}

Atomic contacts determined with the CCP4i implementation of *CONTACT* and a cut-off of 4.5 Å.
Van der Waals interactions defined as non-hydrogen bond contact distances of 4.0 Å or less.
Hydrogen bond interactions are defined as contact distances of 3.3 Å or less.
Salt bridge is defined as contact distances of 4.5 Å or less.
^{G} = contacts with Galactose head group.
^{S} = contacts with Sphingosine chain.
*Not seen = Electron density missing in structure.
Bold = Not observed in NKT TCR-CD1d-α-GalCer complex
Bold = Not observed in NKT TCR-CD1d-C20:2 complex

Supplemental fig 1



Supp Figure 1

Supplemental fig 2