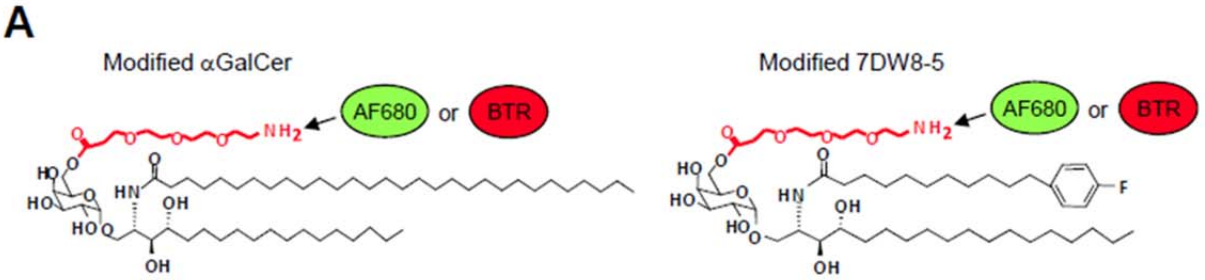
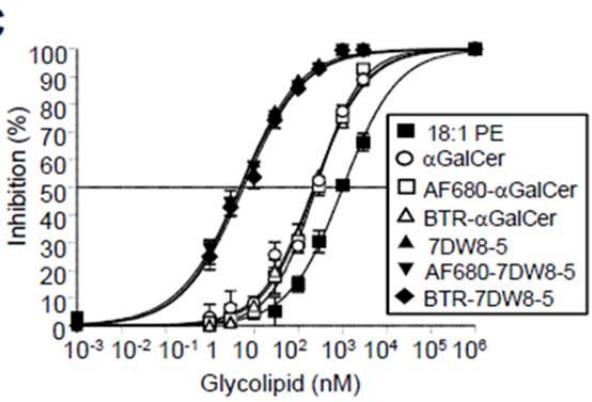
Supplemental Data

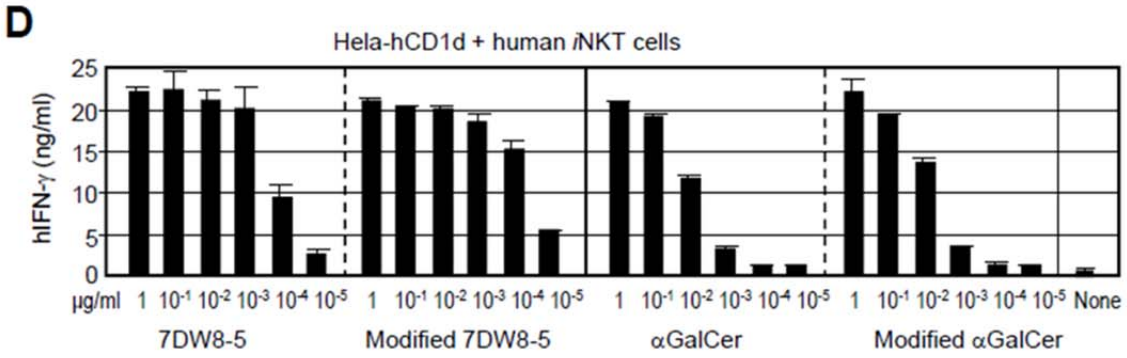
Supplemental Figures

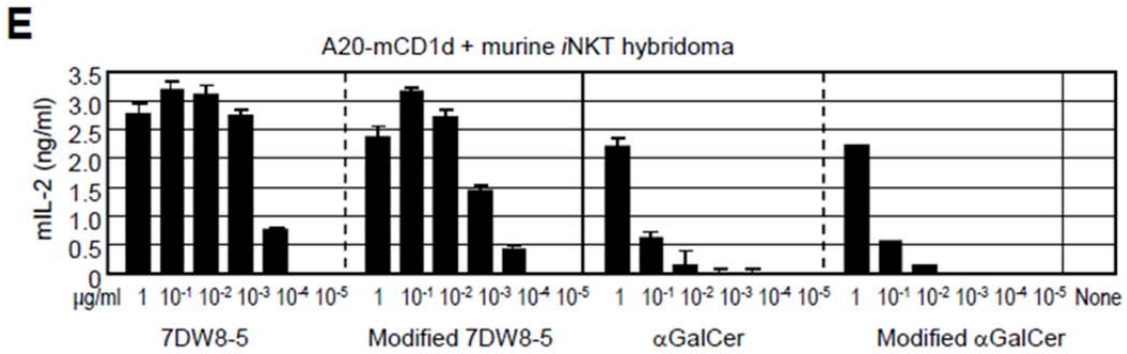


B

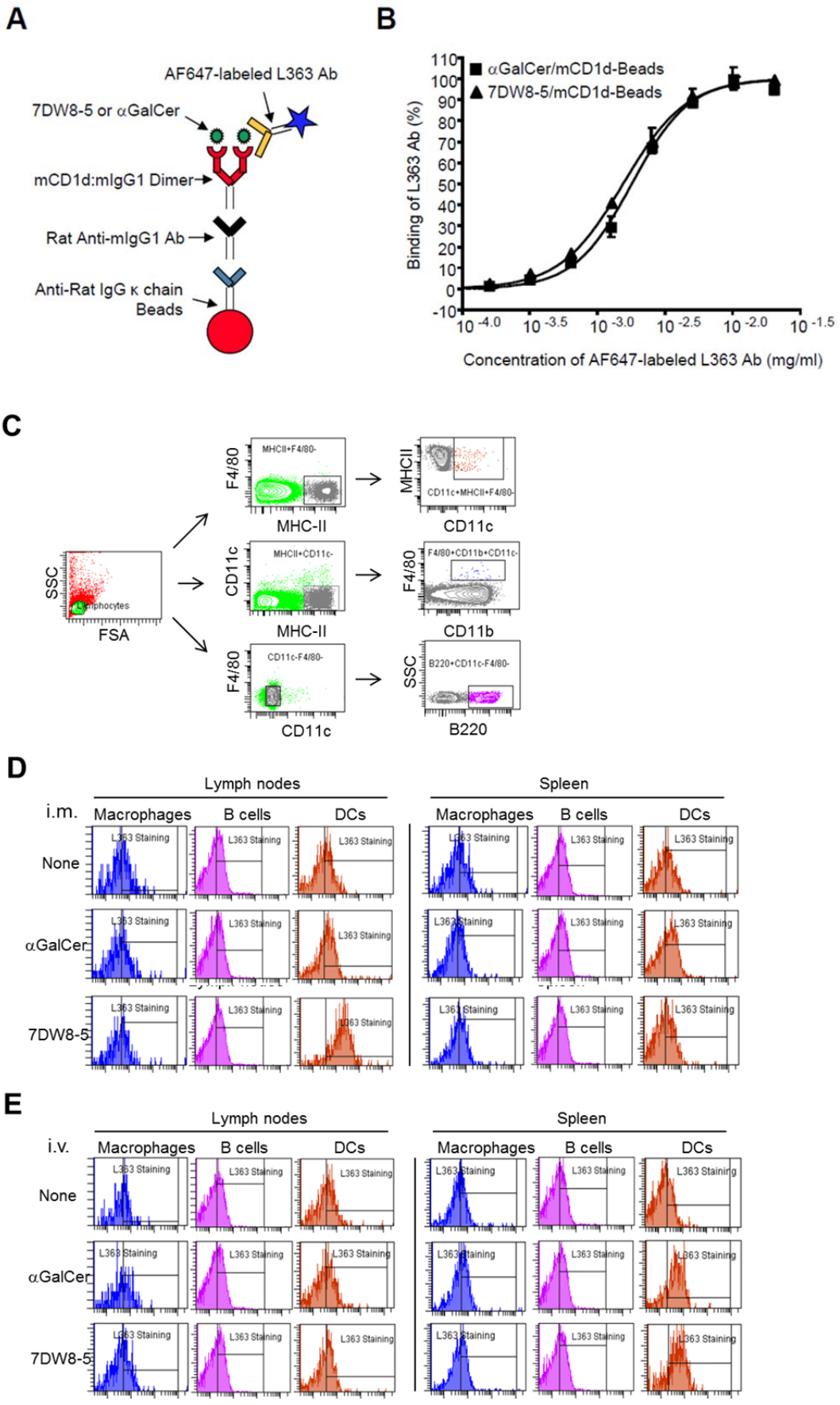
	Calculated MW	Mass spec. MW
AF680-αGalCer	949.9752	949.9756
AF680-7DW8-5	891.8681	891.8686
BTR-αGalCer	1581.0150	1581.0162
BTR-7DW8-5	1464.8021	1464.8029



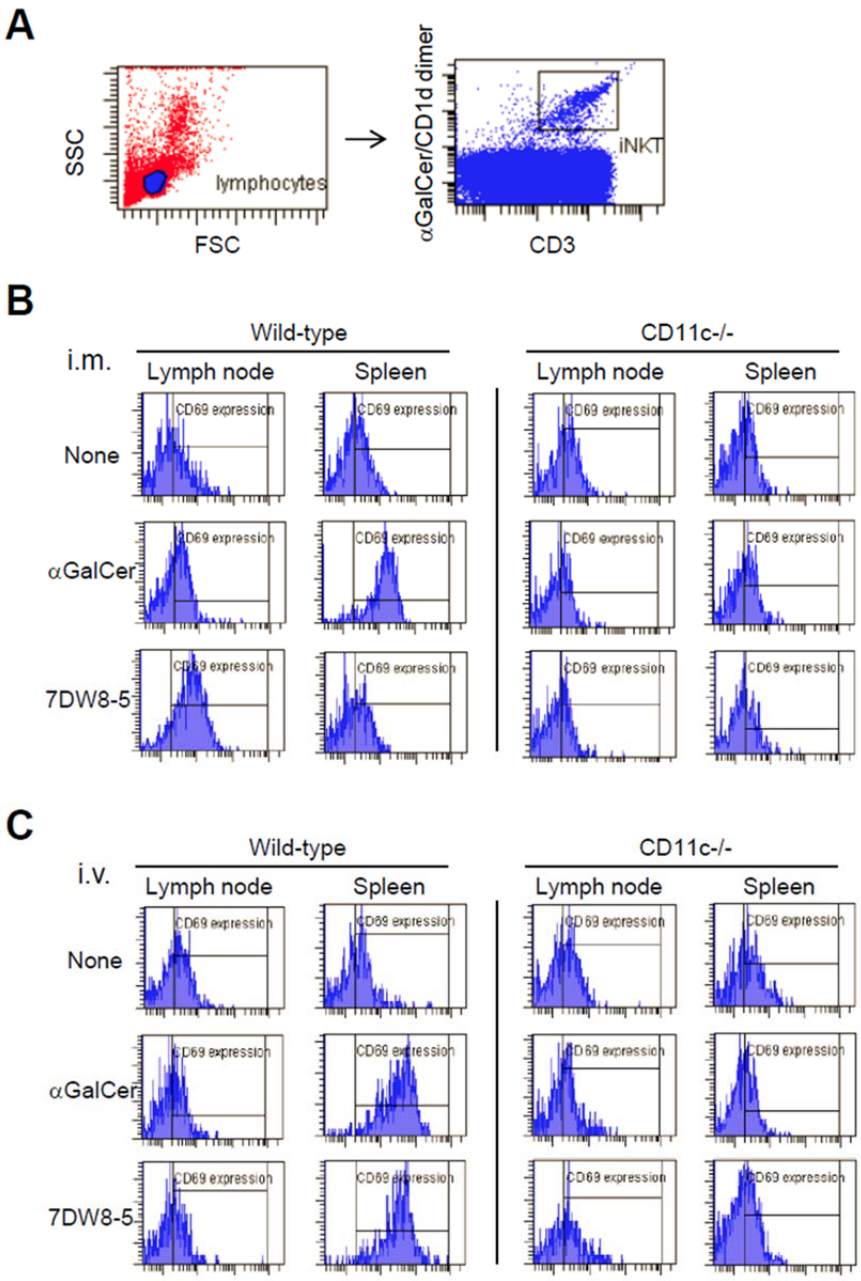




Supplement Fig. 1. Labeling, binding affinity and biological activity of modified glycolipid. (A) Amine-modified αGalCer and 7DW8-5 were synthesized by Aptuit (Greenwich, CT). Succinimide ester of either AF680 or BTR (Molecular Probes) was used to label the modified glycolipids. (B) Labeled glycolipid products were confirmed by mass spectrometry. (C) Affinities between glycolipid analogs and mCD1d molecule were measured by a competitive ELISA. Glycolipid analogs were serially diluted in the presence of 1 μg/ml 18:1 Biotinyl PE lipid, as the competitor. CD1d/biotinyl PE complex was detected with HRP-labeled avidin. 18:1 Caproylamine PE lipid was used as a positive control. Inhibition curves were generated using GraphPad Prism 4.0 software. Mean ± SD of triplicate assays is shown. (D, E) Biological activity of modified glycolipids was compared with that of unlabeled glycolipids. In (D), 2 x 10^4 human *i*NKT cells were co-cultured with 2 x 10^4 Hela cells transfected with a human CD1d gene, or in (E) 2 x 10^4 mouse *i*NKT hybridoma cells were co-cultured with 2 x 10^4 A20-mCD1d cells, in the presence of an indicated concentration of each glycolipid. After 24-hr incubation, the culture supernatants were collected and the concentrations of human IFN-γ and mouse IL-2 in the supernatants were determined by ELISA. Mean ± SD of four triplicate assays is shown.

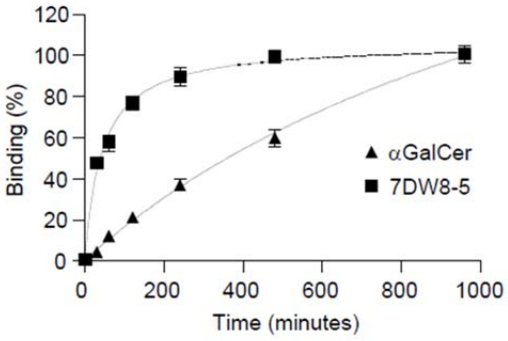

A
AF647-labeled L363 Ab
7DW8-5 or αGalCer
mCD1d:mIgG1 Dimer
Rat Anti-mIgG1 Ab
Anti-Rat IgG κ chain
Beads
B
αGalCer/mCD1d-Beads
7DW8-5/mCD1d-Beads
Binding of L363 Ab (%)
Concentration of AF647-labeled L363 Ab (mg/ml)
C
SSC
FSA
Lymphocytes
F4/80
MHC-II
MHCII+F4/80-
MHCII
CD11c
CD11c+MHCII+F4/80-
CD11c
MHCII+CD11c-
F4/80
CD11b
F4/80+CD11b+CD11c-
CD11c-F4/80-
SSC
B220
B220+CD11c-F4/80-
D
Lymph nodes
Spleen
i.m.
Macrophages
B cells
DCs
None
αGalCer
7DW8-5
L363 Staining
E
Lymph nodes
Spleen
i.v.
Macrophages
B cells
DCs
None
αGalCer
7DW8-5
L363 Staining

Supplemental Fig. 2. Use of L363 antibody, which equally binds αGalCer/mCD1d and 7DW8-5/mCD1d complexes, in order to determine the glycolipid/CD1d complex expressed by various APCs *in vivo*. (A) After incubating 2 μg of rat anti-mouse IgG_1 antibody with anti-rat IgGκ chain antibody coated microbeads for 2 hr, the microbeads were incubated with 1 μg of mCD1d:mIgG1 dimer for 2 hr. After washing, microbeads were loaded with 2 μg of 7DW8-5 and αGalCer at 37^0C overnight. Then, microbeads were stained with serial diluted AF647-labeled L363 antibody for 30 min, and then analyzed with BD LSRII flow cytometer. (B) The binding kinetics of L363 antibody staining was fit using GraphPad Prism 4.0 software. Mean ± SD of triplicate assays is shown. (C) Lymphocytes, isolated from PLNs and spleens of mice 8 hr after i.m. or i.v. injection with glycolipids, were stained with L363 antibody, anti-mouse MHC-II, anti-mouse CD11c, anti-mouse F4/80, anti-mouse CD11b and anti-B220 antibodies. Macrophages were gated as $CD11c^-CD11b^+F4/80^+$, B cells were gated as $B220^+CD11c^-F4/80^-$, and DCs were gated as $CD11c^+MHC\text{-}II^+F4/80^-$. Representative histograms from one mouse receiving glycolipid by (D) i.m. or (E) i.v. are shown.



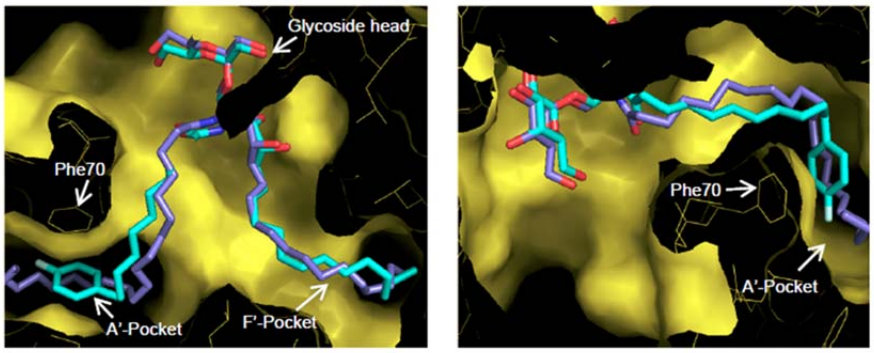
Supplemental Fig. 3. DCs are required for early *i*NKT cell activation. CD11c-DTR mice were administered 100 ng diphtheria toxin by i.p. injection, and 36 hr later, mice were injected i.m. or i.v. with glycolipid. (A) PLNs and spleens were isolated 2 hr after injection, stained with CD3 antibody and αGalCer-loaded mCD1d dimer, and *i*NKT cells were gated by being a double positive. *i*NKT cell activation was assessed by monitoring CD69 expression and the results were quantified by flow cytometry. Representative histograms from one mouse receiving glycolipid by (B) i.m. or (C) i.v. are shown.

A



Glycolipid	$T_{1/2}$ (minutes)
αGalCer	377±22
7DW8-5	23.7±3.9

B



Supplemental Fig. 4. Kinetics and molecular modeling of 7DW8-5 and CD1d binding. (A) CD1d-expressing cells capture 7DW8-5 faster than αGalCer. A20-mCD1d cells (2 x 10^5) were loaded with 2 μg αGalCer or 7DW8-5 at 37^0C. After 0, 15, 30, 60, 120, 240, 480 and 960 min, binding was stopped by fixing the cells with ice-cold paraformaldehyde, and L363 antibody was used to quantify CD1d-bound glycolipid. The results were expressed as mean ± SD of triplicates at each time point. (B) Proposed binding model of 7DW8-5 (cyan), a fluorinated analog of αGalCer, to mCD1d. To generate the model of 7DW8-5 binding CD1d, the coordinates of αGalCer (PDB entry 3TO4) were loaded into the program. MacPyMOL and the Build/Fragment and Sculpting Wizards were used to modify the compound and rearrange positioning to avoid collisions with mCD1d. The yellow surface (PDB entry 3TO4 using protein atoms only) describes the pocket of mCD1d in the presence of αGalCer (blue violet). The assumption is that the crystallization detergent molecule will be displaced by the 7DW8-5, due to lack of space to accommodate the longer aliphatic chain and the substitution of a hydrogen atom for a fluorine atom.