Article

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Abstract

Activation of invariant natural killer T lymphocytes (iNKT cells) by α-galactosylceramide (α-GC) elicits a range of pro-inflammatory or anti-inflammatory immune responses. We report the synthesis and characterization of a series of α-GC analogues with acyl chains of varying length and a terminal benzophenone. These bound efficiently to the glycolipid antigen presenting protein CD1d, and upon photoactivation formed stable CD1d-glycolipid covalent conjugates. Conjugates of benzophenone α-GCs with soluble or cell bound CD1d proteins retained potent iNKT cell activating properties, with biologic effects that were modulated by acyl chain length and the resulting affinities of conjugates for iNKT cell antigen receptors. Analysis by mass spectrometry identified a unique covalent attachment site for the glycolipid ligands in the hydrophobic ligand binding pocket of CD1d. The creation of covalent conjugates of CD1d with α-GC provides a new tool for probing the biology of glycolipid antigen presentation, as well as opportunities for developing effective immunotherapeutics.

Keywords

CD1d, α-galactosylceramide, benzophenone, invariant Natural Killer T cells

Introduction

Invariant Natural Killer T (iNKT) cells are a prominent subset of unconventional T cells that bridge innate and adaptive immunity to contribute to a wide range of immune responses.[1] They recognize and respond to glycolipid antigens presented by CD1d, a membrane protein specialized for binding and presentation of lipid antigens.[2] The most extensively studied CD1d-presented glycolipid antigens are synthetic forms of α-galactosylceramide (α-GC), which potently stimulate iNKT cell proliferation, expansion and cytokine secretion.[3] In mice, various structural analogues of α-GC have shown impressive anti-cancer effects[4], as well as a broad range of activities in models of infection, vaccination, autoimmunity and inflammatory diseases.[5] Thus, there has been increasing interest in using α-GC analogues to develop new approaches to vaccination or immunotherapy.[4c, 6]

Despite the potent immune activating properties of α-GC and the conservation of a CD1d-restricted iNKT cell subset in humans, there has been limited success so far in translating iNKT cell-based approaches into clinical applications. This may reflect problems with systemic delivery of glycolipid agonists, which leads to their uptake and presentation by a wide range of cell types and the stimulation of unpredictable or antagonistic immune responses.[6a, 7]

Several approaches to overcoming these problems have been developed that involve delivery of α-GC by antigen presenting cells (APCs) or soluble recombinant CD1d proteins loaded *ex vivo* with the glycolipid. [8] These approaches have shown potential to stimulate more effective antitumor responses compared to injections of free glycolipids in mouse models, as well as promising preliminary results in

preclinical and clinical studies.[4c, 6a, 8c, 8d, 9] Also of note is the apparent ability of these methods to induce substantial iNKT cell activation while triggering less of the long-term unresponsiveness (anergy) or depletion of iNKT cells that has been a problem with injections of free glycolipids.[10]

However, a potential limitation is presented by the readily reversible binding of glycolipid ligands to CD1d, which is mediated by noncovalent hydrophobic and hydrogen bonding interactions.[2] Rapid dissociation has been reported to result in half-lives for αGC-CD1d complexes in some *in vitro* studies as short as a few minutes or less, which may be further reduced *in vivo* by the presence of lipid exchange and binding proteins.[11] The relatively short half-life and instability of such complexes limits the duration and potency of their desirable biologic effects, and the release of free glycolipids *in vivo* may contribute to unwanted effects such as iNKT cell anergy or toxicities including liver damage or sensitization to endotoxic shock.[12] Thus, the relative instability of αGC-CD1d complexes remains a suboptimal feature in approaches that involve *ex vivo* loading of cells or CD1d proteins with glycolipids, and can compromise the efficacy and precision of such controlled delivery methods.

In the current study, we have developed an approach to circumvent the problem of glycolipid dissociation from CD1d by the use of photoactivatable forms of α-GC to create covalently stabilized and highly active αGC-CD1d conjugates. A series of analogues of α-GC containing a benzophenone group at the terminus of their N-acyl chains was synthesized and optimized for iNKT cell stimulating activity. Controlled exposure to UV irradiation generated stable covalent conjugates of these glycolipids with CD1d, and these retained their ability to potently stimulate iNKT

cells *in vitro* and *in vivo* in mice. Specific immunologic properties such as cytokine production could be modulated by variations in the size of the aliphatic chain bearing the benzophenone group, which was correlated with the affinity of the conjugates for the antigen receptors of iNKT cells. Mass spectrometry identified a unique site for covalent bond formation in the CD1d protein, enabling detailed modeling of the structure of the stable conjugates. The development of controlled covalent bond formation for stabilization of αGC-CD1d conjugates provides a new tool for the study of glycolipid antigen presentation, and also forms the basis for improving immunotherapies based on the targeted delivery of iNKT cell activators.

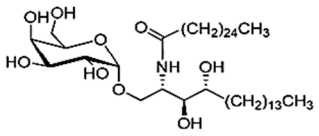
Results

Synthesis of BPGCs

Extensive previous work has shown that many modifications of the fatty acyl chain of α-GC can be tolerated without disruption of glycolipid binding to CD1d or loss of iNKT cell stimulating activity[3], consistent with the large volume of the hydrophobic ligand binding site of CD1d.[2, 13] Thus, we developed a synthetic strategy for introduction of a photoactivatable moiety on the acyl chain terminus of α-GC to enable the controlled formation of a covalent bond between the glycolipid and the CD1d protein (**Fig. 1**). The benzophenone group was chosen as it can be activated by UV irradiation to give the corresponding benzhydril biradical, which we postulated would react with a proximal C-H bond present in the CD1d protein to form a permanent covalent bond. A group of benzophenone-containing derivatives of α-GC (BPGCs) bearing acyl chains of various lengths (compounds **9**-**15**) were synthesized to determine which would optimally

associate with CD1d and effectively activate iNKT cells. Based on the resemblance of benzophenone to a C10 isoprene unit, we synthesized a range of BPGCs which mimic *N*-acyl chain lengths from C16 (C6:BP (**9)**) to C26 (C16:BP (**15)**), thus spanning the range found in most highly active α-GC analogues.[3] These compounds were readily prepared via acylation of the parent compound **1** with carboxylic acids (**2**-**8**), following their conversion to the corresponding acid chlorides using oxalyl chloride. Ensuing acylation of the amine **1** in a 1:1 mixture of THF and saturated sodium acetate solution afforded the benzophenone-derivatised glycosphingolipids (BPGCs) **9-15** (**Fig. 1**).

a



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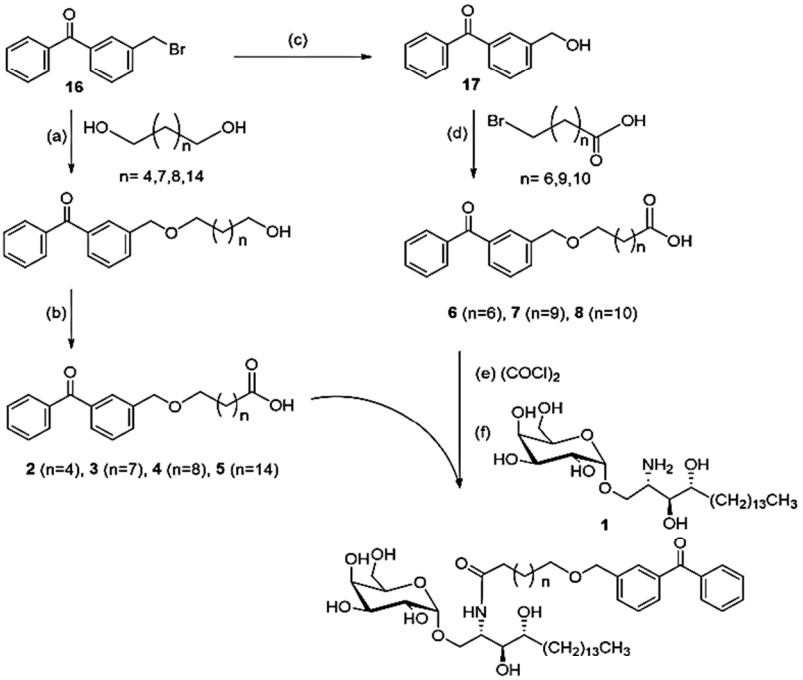


Figure 1: Synthesis of BPGCs. (a) Structure of the prototypical iNKT cell activating glycolipid, α-GC C26:0. **(b)** General scheme for synthesis of BPGCs with acyl group spacers of varying length (compounds **9 – 15**). (a) NaH, DMF, 0 ºC to rt; (b) PDC, wet THF, rt; (c) $CaCO_3$, THF, H_2O, 100 ºC, 12 h; (d) NaH, DMF, 0 ºC to rt; (e) $(COCl)_2$, 60 ºC, 2 h; (f) NaOAc, THF, rt, 12 h. Incorporation of the benzophenone group into carboxylic acids was accomplished through the use of a flexible ether linkage to allow rotational freedom and optimal orientation of the aromatic group in the CD1d ligand-binding pocket. To synthesize carboxylic acids (**2-8**), we used a versatile strategy involving an S_N2 displacement of a bromide using various diols and 3-(bromomethyl)benzophenone (**16**) or through a variety of bromocarboxylic acids by 3-(hydroxymethyl)benzophenone (**17**). The monoalkylation of the diols with 3-(bromomethyl)benzophenone (**16**), which was obtained using published procedures[14], was achieved in reasonable yields by using the diols in excess. Oxidation of the corresponding alcohols to the acids with pyridinium dichromate (PDC) was sluggish and only afforded compounds **2-5** in average yields. In contrast, alkylation of the alcohol **17** with the bromocarboxylic acids in DMF and sodium hydride as base proceeded smoothly to afford the desired carboxylic acids **6-8** in quantitative yields (see Supplemental Information for further details of synthesis and characterization).

Stimulation of iNKT cells by BPGC analogs

A variety of *in vitro* biological assays were performed to assess the iNKT cell activating properties of the BPGCs upon presentation by CD1d, and to determine the effect of the varying acyl chain lengths and the presence of a bulky terminal benzophenone group on their biologic activities. A standard assay using co-culture of mouse bone marrow-derived dendritic cells (BMDCs) and a murine iNKT cell hybridoma was used to assess the activity and relative potencies of BPGCs (**Fig. 2a**).[15] This showed significant iNKT cell stimulating activity for all BPGCs tested, with a substantial impact of the length of fatty acyl chain. Optimal iNKT stimulation in the mouse cell culture system was

achieved with **13**, which along with several other BPGCs was significantly more potent than the standard α-GC (C26:0), which is generally considered a highly potent iNKT cell activator both *in vitro* and *in vivo*.[4a, 15] A similar *in vitro* analysis was carried out using a canonical human iNKT cell clone co-cultured with HeLa cells transfected to express human CD1d (**Fig. 2b**).[16] This also revealed strong activity of BPGCs as measured by IFN-γ secretion, which was similar to or greater than that stimulated by C26:0. As for the mouse culture system, compounds **12** and **13** showed the greatest potency in this assay, although length of the fatty acyl tail had much less apparent impact in the human system.

To directly assess activity *in vivo*, we analyzed serum cytokine levels following intravenous injection of selected BPGCs. For this analysis, we tested **9** which was a relatively weak activator of murine iNKT cells *in vitro*, and **13** and **15** which were more potent than C26:0 in both mouse and human cell culture assays. Mice were injected with 4 nmoles of each glycolipid and bled after 2, 10 and 24 hours to quantitate serum IFN-γ and IL-4, as previously described.[15] Significant levels above baseline for serum IL-4 were detected with **13** and **15** at two hours, which declined to undetectable levels by 10 hours (**Fig. 2c**). The IL-4 levels were several fold higher for **13** and **15** compared to C26:0, indicating a rapid and powerful activation of iNKT cells. Consistent with this, IFN-γ levels showed a sustained rise with **13** and **15** with a peak at 10 hours that closely resembled the response to C26:0, while **9** did not stimulate detectable levels above background for either cytokine tested. Thus, BPGCs retained their iNKT cell activating properties *in vivo*, and the potencies of different analogues varied depending on the length of their acyl chains.

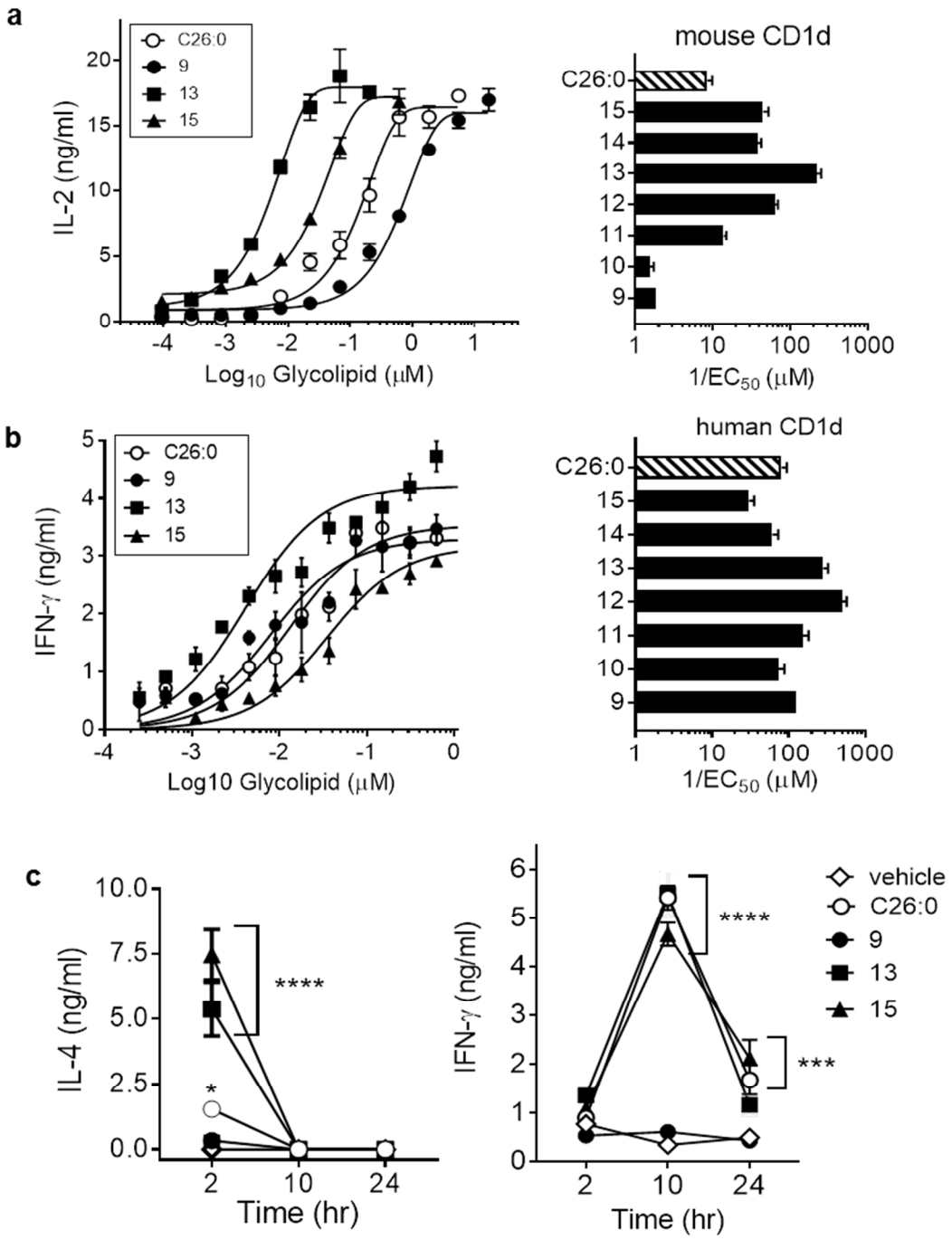


Figure 2: iNKT stimulatory activity of BPGCs. (**a**) Responses of mouse iNKT cell hybridoma DN3A4-1.2 cultured with bone marrow derived dendritic cells from C57BL/6 mice and the indicated concentrations of BPGCs or α-GC C26:0. IL-2 secretion was measured in supernatants after 18 hours of culture. Representative dose-response curves are shown on the left for three of the seven BPGCs and standard α-GC C26:0.

Concentrations stimulating 50% of the maximum response (EC_{50}) were determined for each glycolipid, and reciprocal values are plotted on the right. (**b**) Similar analysis as in (**a**) except using human iNKT clone HDE3 and HeLa cells expressing human CD1d with measurement of secreted IFNγ as the readout for activation. (**c**) *In vivo* activities of **9**, **13** and **15** were determined by quantitating serum IL-4 and IFN-γ 2, 10 and 24 hours post glycolipid injection. Peak values for serum IL4 (left) and IFN-γ (right) were found at 2 and 10 hours respectively.

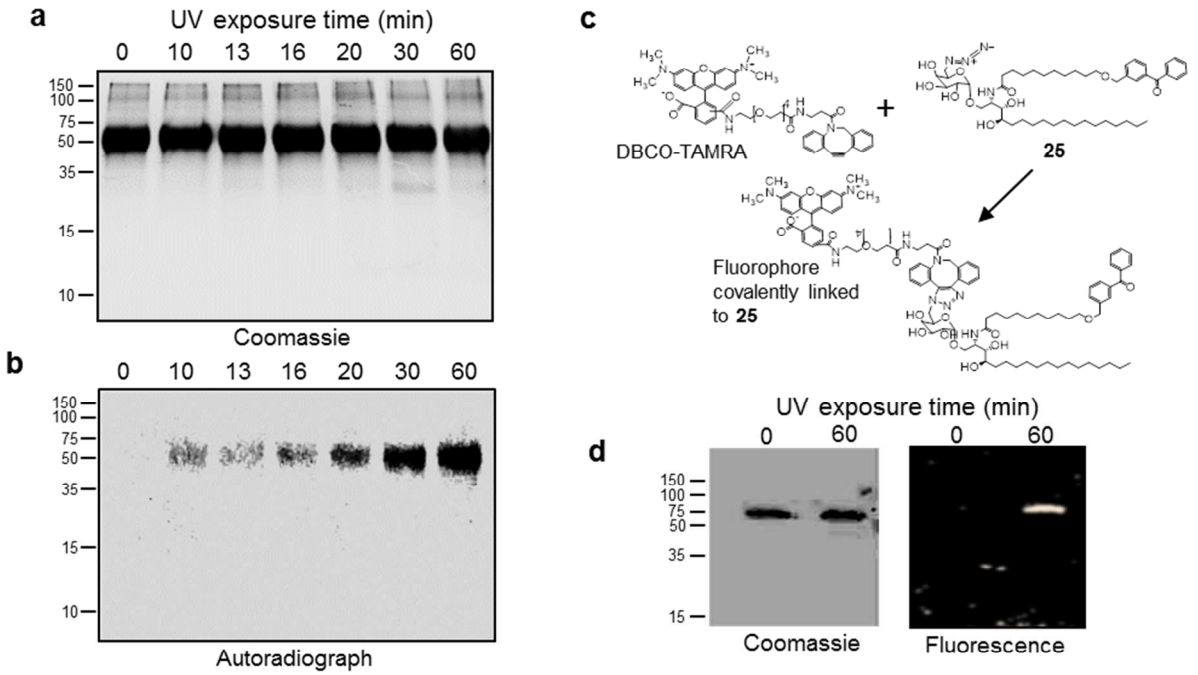
All symbols and bars are means for triplicate samples, and error bars are ±1 SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 for comparisons to vehicle treated mice (two-way ANOVA with Dunnett's multiple comparison test). All data are representative of at least three separate experiments.

Formation of covalent CD1d-glycolipid conjugates by photoactivation

The known photochemical properties of benzophenones predicted that loading of BPGCs into CD1d proteins followed by exposure to UV irradiation should form covalently stabilized protein-glycolipid conjugates. We validated this initially using a 3-fold molar excess of ^{14}C-labelled analogue of **13**, compound **26** (Scheme 2, SI) to load soluble recombinant mCD1d protein. Aliquots of the loaded protein were then exposed to a 365 nm UV lamp for times ranging from 0 to 90 minutes, followed by denaturation (1% SDS at 100° C for 5 min) and separation by SDS-PAGE. Staining of the gel with Coomassie blue revealed intact protein in all samples, which ran at the predicted size of ~57 kDa for monomeric soluble CD1d (**Fig. 3a**). Autoradiography of the same gel revealed the presence of the ^{14}C label co-migrating with CD1d protein in the samples exposed to UV light, but not in the unexposed sample (**Fig. 3b**). Maximum association of radiolabel, indicating the formation of stable protein-glycolipid conjugates that were resistant to denaturation, was achieved following 30 to 60 minutes of UV exposure,

corresponding to a delivered dose range of 400 – 600 mJoules/cm^2. Radiometric analysis showed that approximately 70% of CD1d molecules were conjugated to ^{14}C labeled **26** after 60 minutes of UV exposure (**Supplemental Figure S1**).

1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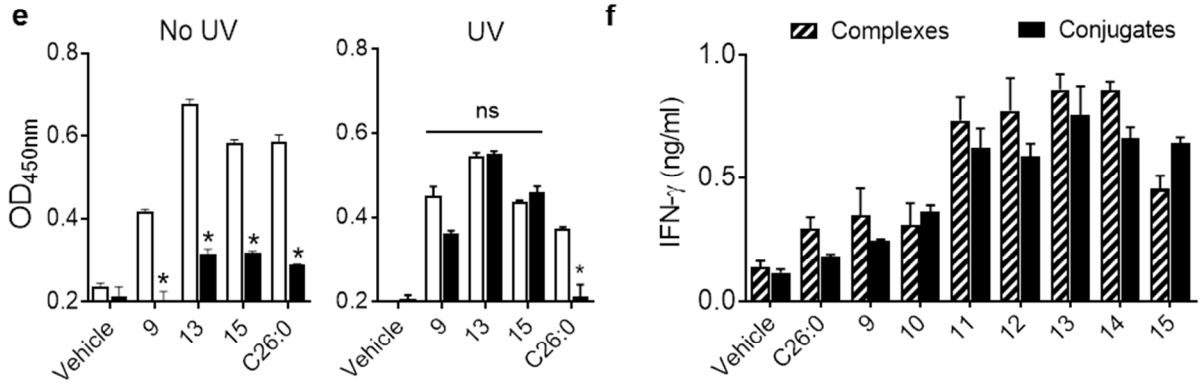
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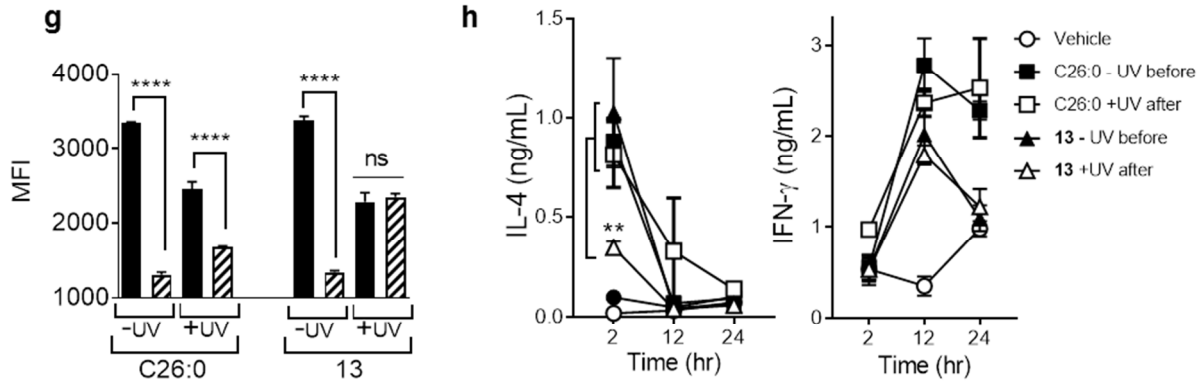
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Figure 3: Covalent conjugation of BPGCs to mCD1d. (**a**) Coomassie stained gel and **(b)** autoradiograph of denaturing SDS-PAGE after exposing mCD1d: ^{14}C-labelled analogue of **13**, compound **26** (Scheme 2, SI) complexes (~57 kDa) for various lengths of time. **(c)** Diagrammatic representation of click reaction of DBCO-TAMRA dye with azide-linked-**25** (SI) either loaded non-covalently or covalently conjugated to CD1d protein. **(d)** Coomassie stained and fluorescent images of denaturing SDS-PAGE gel of fluorescently tagged noncovalent-complexes (0 min UV exposure) and covalent-conjugates (60 min UV exposure) of mCD1d fusion protein (~78 kDa). (**e**) Complexes (No UV, left) or conjugates (UV, right) loaded with the indicated BPGC or α-GC-C26:0 were coated on high binding plates and incubated for 3 days at room temperature either without (white bars) or with (black bars) washing twice per day with PBS + 0.1% Triton X-100. Residual glycolipid binding to mCD1d was detected by ELISA using biotinylated monoclonal antibody L363. $^{*}P < 0.001$ for multiple t tests of comparisons of washed versus unwashed samples with each glycolipid. (**f**) Splenocytes (2 x 10^5 per well in 0.2 ml complete medium) were stimulated *in vitro* for 18 hours with complexes (hatched bars) and conjugates (solid bars) immobilized on high binding plates. Supernatants were collected and assayed for IFN-γ by ELISA. (**g**) JAWS II cells (5 x 10^4 per well) pulsed overnight with 100 nM glycolipids (C26:0 or **13**) were either exposed to UV (~400 mJ/cm^2) or left untreated. Cells were stained with L363-AlexaFluor647 either directly (solid black bars) or after multiple washes (hatched bars) to allow dissociation and analyzed by flow cytometry. $^{****}P < 0.0001$, two-way ANOVA with Bonferroni correction for indicated comparisons. (**h**) JAWS II cells were exposed to UV (600 mJ/cm^2) either before (black symbols) or after (white symbols) pulsing with vehicle, C26:0 or **13** at 100 nM concentration. Cells were washed thrice during 24 hours of incubation and adoptively transferred i.v. into mice (3 x 10^5 cells per mouse, 4 mice per group). Blood samples were obtained after 2, 12 and 24 hours to quantitate serum IL-4 and IFN-γ. $^{**}P < 0.01$, two-way ANOVA with Bonferroni correction for indicated comparisons. Data plotted in (**e**) – (**f**) are shown as means for a minimum of three replicates, and error bars are ± 1 SD. All experiments were performed at least three times.

Further analysis of conjugate formation was carried out in studies using compound **25** (synthesis described in SI), an analogue of **13** carrying an azido group at the 6'- position of the saccharide head group (**Fig. 3c**). Complexes formed between this glycolipid and soluble mCD1d were either irradiated with UV λ365 or not, and then incubated with fluorescent DBCO-TAMRA, which reacts with the azido group of the glycolipid.[17] The samples were then denatured and analyzed by SDS-PAGE. While gels stained with Coomassie blue showed similar CD1d protein in both samples (migrating at ~78 kDa, consistent with the mCD1d fusion protein used for this experiment; see Online Methods for details), only the sample exposed to UV had a fluorescent signal co-migrating with CD1d (**Fig. 3d**). This confirmed the formation of stable conjugates following photoactivation of the benzophenone moiety in CD1d-glycolipid complexes. In addition, the ability of the DBCO-TAMRA reagent to couple to the azido group was consistent with correct orientation of the **13** in the CD1d lipid binding groove, with the carbohydrate head-group exposed and accessible at the surface of the protein.

To confirm the correct conformation and stability of covalent mCD1d-BPGC conjugates, we tested reactivity with mAb L363, which binds specifically to CD1d loaded with α-GC in a manner that closely mimics the TCRs of iNKT cells.[18] Plate immobilized mCD1d proteins were loaded with BPGCs, then either UV irradiated or not and tested for binding of L363. This showed binding to levels comparable to that with C26:0 loaded mCD1d (**Fig. 3e**). Furthermore, after repeated washing with buffer containing mild detergent to remove reversibly bound glycolipids, we observed significant loss of L363 binding in samples without UV irradiation. In contrast, UV exposed samples

loaded with BPGCs showed no significant loss of L363 binding, indicating covalent bond formation. As expected, L363 binding to mCD1d loaded with C26:0 was reversible under these conditions with or without UV exposure. To further characterize the biologic activity of these complexes or conjugates, we also assessed their ability to stimulate iNKT cell activation in mouse splenocytes (**Fig. 3f**). The UV treated stable conjugates retained their iNKT cell stimulating activity at levels comparable to noncovalent complexes, indicating that UV exposure and covalent bond formation did not adversely alter TCR recognition. Analysis of all seven BPGCs in this assay showed an influence of the acyl chain spacer length on potency of stimulation, with **13** generally showing the strongest stimulation.

In addition to the analyses of loading and photo-crosslinking to purified recombinant CD1d proteins, we also assessed the formation of stabilized mCD1d-glycolipid conjugates on intact CD1d-expressing antigen presenting cells. We used the murine immortalized dendritic cell line JAWS II for this, since it expresses mCD1d and is capable of glycolipid antigen presentation.[19] Incubation of these cells with either C26:0 or **13** generated strong surface staining with mAb L363, which for both glycolipids was greatly reduced following incubation for 1 day in the absence of the glycolipids. In contrast, exposure of the cells to UV irradiation following culture with the **13** eliminated any loss of L363 staining with subsequent culture, whereas UV irradiation of C26:0 loaded cells did not prevent decay of L363 binding under the same conditions (**Fig. 3g**). This strongly suggested that stabilized covalent CD1d-glycolipid conjugates were produced by UV photoactivation of BPGCs in living cells. This was further evaluated by testing the ability of JAWS II cells loaded with **13** and UV treated to stimulate iNKT cell

responses *in vivo* following adoptive transfer of the cells into mice (**Fig. 3h**). We detected strong serum cytokine responses in mice receiving cells bearing the putative covalently stabilized conjugates, and a reduced level of IL-4 relative to IFN-γ was observed when compared to injection of cells presenting noncovalent complexes (i.e., JAWS II cells without UV photoactivation or loaded with C26:0).

Impact of conjugation on TCR affinity and biologic activity *in vivo*

By eliminating dissociation of glycolipid binding to CD1d, we anticipated that conjugation should increase the overall affinity of cognate interactions with iNKT cell TCRs. To assess this, fluorescent tetramers of soluble mCD1d loaded with C26:0 or BPGCs were prepared with and without covalent crosslinking. Binding avidities of tetramers to the TCRs of mouse iNKT cell hybridoma line DN3A4-1.2 were quantified by measuring equilibrium binding to the cells over a range of concentrations, as previously described.[15] Extrapolation of the equilibrium dissociation constant (K_D) values showed maximum avidities for C26:0 and **15** loaded noncovalent complexes, while avidities declined progressively for BPGCs with shorter acyl tail spacers (**Fig. 4a**). This trend was not evident with the binding of covalent conjugate tetramers, as the conjugates with shorter acyl chain variants of BPGCs showed enhanced and more uniform avidities. These results indicated that dissociation of the glycolipids with shorter acyl tails had a major impact in TCR avidity, which was reversed by covalent stabilization of the glycolipid-protein interaction. One apparent exception was the **9** conjugate, which showed a distinctly lower TCR avidity than the other conjugates tested despite covalent stabilization.

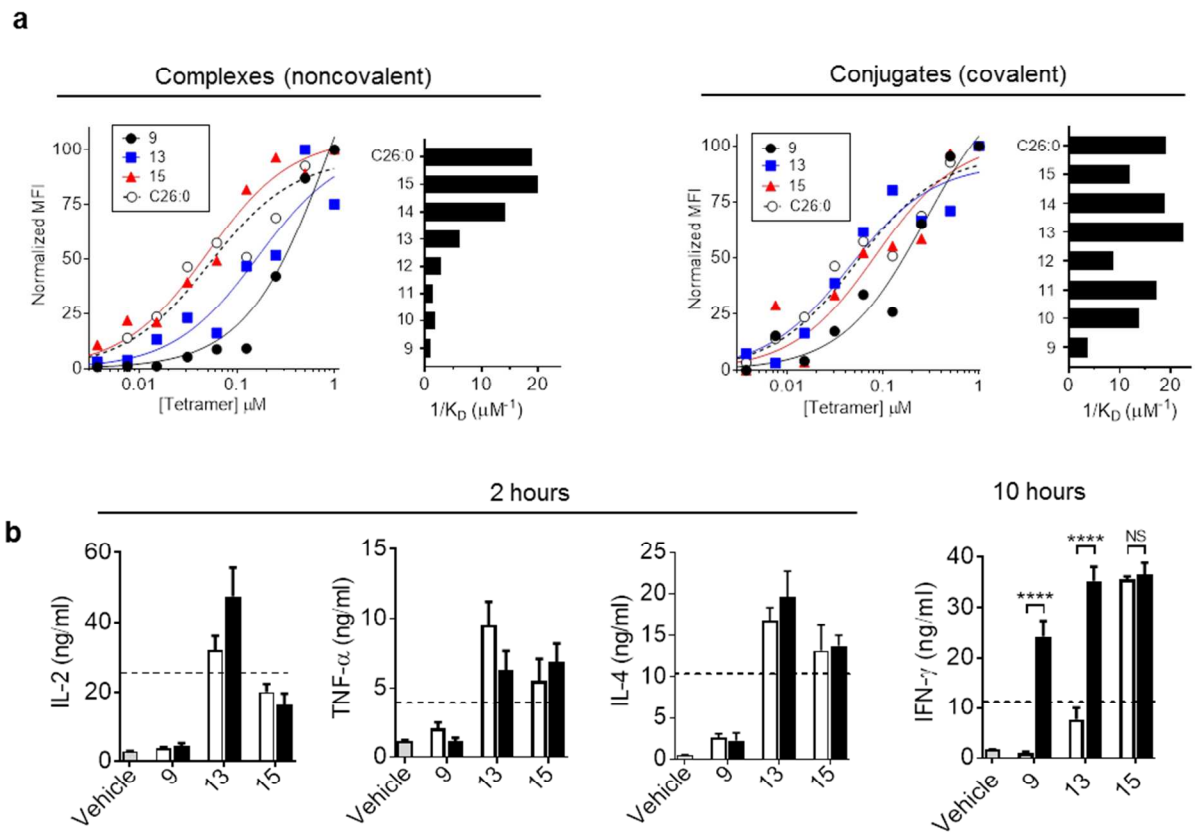

Figure 4: Affinity for iNKT cell TCR and effects on cytokine profiles. (**a**) Equilibrium binding of mCD1d tetramers loaded with BPGCs over a range of tetramer concentrations was used to assess avidities of complexes (not UV irradiated, left) and conjugates (UV irradiated, right) for TCRs of mouse iNKT cell hybridoma DN3A4-1.2. Cells stained with tetramers for 1 hour at room temperature were analyzed by flow cytometry. Normalized representative binding curves are shown for three BPGC loaded tetramers, and for standard C26:0 loaded tetramers (not UV irradiated) for comparison (dashed lines). Bar graphs show reciprocal of K_D values ($1/EC_{50}$) to summarize results for all tetramers. (**b**) Serum cytokine levels following i.v. injection of mice with complexes (open bars) or conjugates (filled bars) loaded with the indicated BPGCs. Serum levels are shown at 2 hours post injection for IL-2, TNF-α and IL-4, and at 10 hours for IFN-γ. Background levels of cytokines in sera from mice injected with inert aqueous vehicle (gray bars), and levels for mice injected with 4 nmoles of free C26:0 glycolipid (horizontal dashed lines) are shown for reference. Bars represent means for groups of 4 animals each, and error bars show 1 SD. ****$P < 0.0001$ for conjugate

versus complex in the indicated comparisons (2-way ANOVA with Bonferroni's multiple comparisons test). NS, not significant ($P > 0.05$). Differences were not significant for other comparisons shown between complexes and conjugates.

We next assessed the *in vivo* activities of soluble mCD1d-BPGC complexes and conjugates using three different BPGCs that varied in their affinities for iNKT cell TCRs. After a single i.v. injection of mCD1d complexes or conjugates loaded with **9**, **13** or **15** (30 μg of mCD1d protein containing ~0.4 nmoles of each glycolipid), serum levels of IL-2, TNF-α, IL-4 and IFN-γ were determined at 2, 10 and 24 hours after administration (**Fig. 4b, and Supplemental Fig. S2**). Injection of C26:0 as a free glycolipid was used as a standard which is known to activate iNKT cell-dependent release of various cytokines, such as IL-2, TNF-α and IL-4 which peak in serum at approximately 2 hours, and IFN-γ which peaks at 10-12 hours after the injection.[20] The administration of conjugates formed with **13** or **15** activated secretion of all four cytokines, including levels of IL-2, TNF-α and IL-4 at 2 hours and IFN-γ at 10 hours, that equaled or exceeded those stimulated by free C26:0. Noncovalent complexes gave similar stimulation as conjugates at 2 hours, but the level of IFN-γ at 10 hours was significantly higher with conjugates for **13**, and also for conjugates with **9** which did not stimulate any detectable serum cytokines at 2 hours. These findings confirmed the iNKT cell activating properties of soluble complexes and conjugates *in vivo*, and also were consistent with the ability of covalent conjugation to stabilize the shorter **9** and **13** glycolipids, extending the duration of their bioactivity. In addition, covalently stabilized **9** conjugates showed a remarkable skewing of the cytokine response such that only IFN-γ was detected among the cytokines measured.

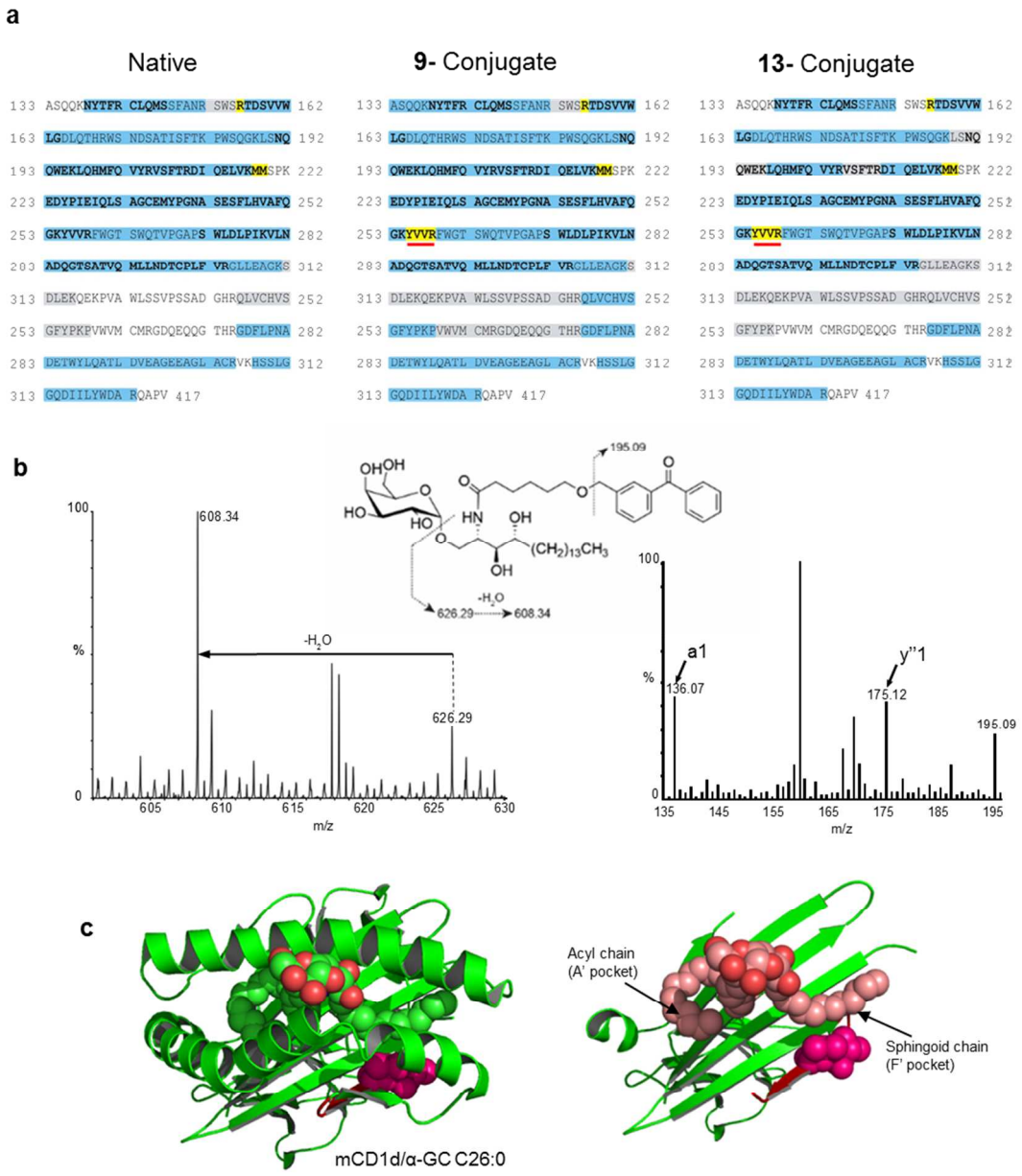

Figure 5: Mapping of covalent attachment site. (**a**) Peptide mapping summaries from nano-LC-ES-MS^E analyses are shown for unloaded mCD1d fusion protein (native) and for protein-glycolipid conjugates containing either **9** or **13**, focusing on the lipid binding region comprising the α1 and α2 regions of CD1d. Amino acid sequences of mCD1d α1 through α2 domains are shown in single letter code. Bold text indicates residues forming the lipid binding pocket, blue shading indicates residues mapped with a high degree of confidence, and grey shading indicates residues mapped with a good degree of confidence. Residues highlighted in yellow indicate amino acids that were not

observed in any detected peptides. Peptides containing the four amino acids underlined in red in both conjugates were detected with high confidence in the native protein but, were absent in both conjugates. (**b**) MS^E analysis of the doubly charged ion m/z 661.33 observed at 30.9 min elution time in the analysis of a reduced, carbamidomethylated, PNGase-F treated tryptic digest of the **9** conjugate. The molecular ion is consistent with the tryptic peptide containing the $_{255}$YVVR_{258} residues modified by a single C6 glycolipid moiety. Labelled ions in the left panel show loss of water from a cleaved C6 BPGC entity, while the right panel highlights evidence for the peptide with the y''1 ion for the C-terminal arginine at m/z 175 and the a1-ion for the N-terminal tyrosine at m/z 136. The ion at m/z 195 is consistent with loss of the benzophenyl group from the BPGC molecule. (**c**) The mCD1d protein structure previously deduced from X-ray crystallography (PDB number 3HE6) is shown as a ribbon diagram in green, and the two valine residues comprising the proposed covalent attachment site are shown as bright pink spheres. The view on the left shows the intact α1 and α2 domains with α-GC C26:0 (green and red spheres) bound in the lipid binding groove. In the view on the right, the α-helices forming the roof of the groove have been removed, and the bound glycolipid is shown as pink and red spheres.

Analysis of conjugation site by peptide mapping

Peptide mapping analyses of samples of native mCD1d and mCD1d conjugates with **9** and **13** were performed to identify possible sites of conjugation. Samples with or without enzymatic deglycosylation were digested with trypsin, or with trypsin plus chymotrypsin. Digested samples were analysed by online nano-liquid chromatography-electrospray tandem mass spectrometry (nano-LC-ES-MS^E) (**Supplemental Fig.S4**). For native mCD1d samples, peptides covering the entirety of the glycolipid binding domains were consistently mapped with a high degree of confidence for all but three residues (R156, M218 and M219) of the putative binding site. Both the **9** and **13** conjugated samples produced very similar data to that of the native mCD1d, with the notable exception that

ions covering the unmodified region $_{255}$YVVR$_{258}$ were not observed (**Fig. 5a**, and **Supplemental Tables S1 – S3**). Instead, molecular ions consistent with the presence of a **13** or **9** modification were detected (**Supplemental Figures S4** and **S5**), and fragmentation of these modified peptides produced signals corresponding to both the *N*-terminal tyrosine and the C-terminal arginine, as well as y"- and b-ions consistent with modified VVR and YVV (**Fig. 5b**). Although definitive evidence for covalent modification of a specific residue was not obtained, these data strongly supported the site of conjugation to be in the region $_{255}$YVVR$_{258}$, with one or both valine residues at the base of the F' pocket of mCD1d being directly involved in the formation of potential bioconjugation products (**Fig. 5C and Supplemental Fig. S6**).

Modelling BPGC analogue binding modes.

To confirm the feasibility of the putative conjugation site identified in mCD1d, the binding modes of **9** and **13** were modelled into the published crystal structure of the ternary complex of CD1d with α-GC C26:0 and the Vα14-Vβ8.2 iNKT cell TCR (PDB entry code 3HE6) (**Fig. 5c and Fig. 6**). Briefly, compound **13** was docked without its benzophenone group, which was deleted prior to docking. The binding of the modified benzophenone group (i.e., diphenylmethanol, formed by the benzophenone group after conjugation) was predicted in a separate docking run considering that the flexible linker in the BPGC analogues would only minimally restrain possible binding conformations of the end group. The most favorable docking pose showed excellent shape complementarity and non-polar contacts in the binding site while linked covalently to the V257 side chain in the F` pocket. The diphenylmethanol pose and the sugar moiety of

13 docked without the benzophenone end group were then connected by predicting an optimal conformation for the flexible linker between them through docking the linker segment (with constraints). Models of the full **9** and **13** structures were generated through splicing in the appropriate linkers of the two BPGC analogues, and were then refined using Prime complex structure refinement in Schrödinger software (**Fig. 6**).

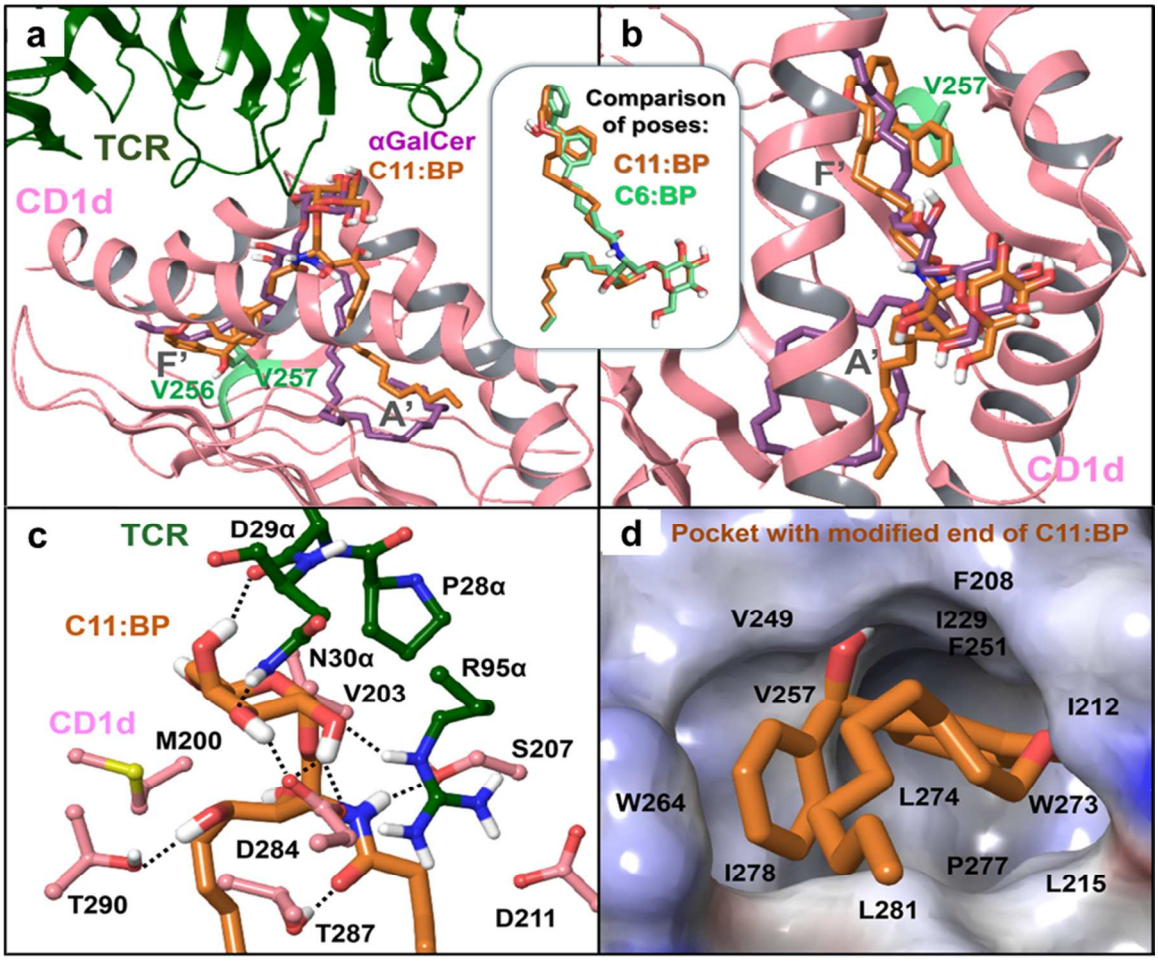

Figure 6: Molecular docking of 13 and 9 in mCD1d. (**a, b**) Two views of the binding model of **13** and co-crystallized α-GC (from PDB code 3HE6). The inset compares the binding models of **13** (C11:BP) and **9** (C6:BP). The modified end in the F` pocket is covalently attached to V257 (residue V125 in the structure with PDB code 3HE6). (**c**) Interactions near the sugar moiety of **13**. A TCR interaction shared between α-GC C26:0 in the crystal structure and both **9** and **13** models was the hydrogen-

bonding interaction of the galactose 3''-OH with N30α. Additional stabilizing interactions with the sugar moiety of the bound BPGCs included hydrogen bonds with TCR residues R95α and D29α and with D284 of mCD1d. The binding site that in the crystal structure is occupied by the vicinal diol of the phytosphingosine chain of α-GC was occupied in the BPGC bound models by a less bulky amide group. This allowed reorientation of the TCRα R95 side chain (R95α) into the same region, forming a favourable stacking interaction between the guanidinium of the arginine and the amide of the ligand. The guanidinium of R95α was also sandwiched between the mCD1d residues D284 and D211, salt-bridging with both aspartates. Other interactions stabilizing the predicted BPGC binding orientation were apparent, such as hydrogen bonds between the amide group and residues S207 and T287 of mCD1d, and between the C3 hydroxyl of the phytosphingosine chain and residue T290 of mCD1d. (**d**) CD1d surface in the region of the modified terminal, colored by electrostatic potential (blue indicates nonpolar and red indicates polar surfaces). The end group forms favourable non-polar contacts with I229, V249, F251, W264, W273, L274, I278 and L281 (residue numbers are based on the mCD1d-fusion protein).

The final refined structures of **9** and **13** glycolipids after docking showed similar binding modes except for the linker region, as expected (**Fig. 6, inset**). Compared to the corresponding sugar moiety of α-GC C26:0 in the crystal structure, the α-galactopyranosyl group in BPGC analogues was positioned closer to the TCR and slightly shifted (**Fig. 6a**). The lipid chains occupying the A' and F' pockets in the models with bound BPGCs were switched compared to the orientation of α-GC C26:0 in the crystal structure. Thus, the acyl chain of the covalently bound **9** and **13** was positioned in the F' pocket of mCD1d, rather than in the A' pocket as in the case of the noncovalently bound α-GC C26:0 (**Fig. 6a and b**). This binding orientation allowed reorientation of the TCRα R95 side chain (R95α) to form a favourable stacking

interaction between the guanidinium of the arginine and the amide of the ligand, as well as a number of other stabilizing interactions (**Fig. 6c**). The acyl chains of **9** and **13** adopted quite similar orientations in the two BPGC models in spite of their different lengths, linking the diphenylmethanol moiety covalently bound to the side chain of V257 in a mainly non-polar pocket (**Fig. 6d**).

Discussion

In this study we synthesized a series of benzophenone-modified glycolipids that can be covalently cross-linked to soluble as well as surface expressed CD1d to generate highly stable conjugates that activate iNKT cells *in vitro* and *in vivo*. By appending a photoactivatable benzophenone group to the end of the amide linked acyl chain of α-GC, we generated a family of BPGCs with varying aliphatic spacer lengths. Screening for biologic activity using a variety of assays for iNKT cell stimulation showed that all of these BPGCs could be presented by CD1d, with most experiments showing **13** to be optimal for iNKT cell stimulation. Using relatively brief and low energy UV irradiation, all of the BPGCs could be activated to form stable conjugates with purified CD1d proteins. In addition, UV photoactivation could be shown to stabilize BPGC interactions with CD1d expressed in living cells. By multiple criteria, UV induced covalent conjugates of BPGCs retained their interactions with iNKT cell TCRs, and conjugates in either soluble, surface bound or cell associated forms possessed the ability to activate cytokine secretion by iNKT cells.

Our primary goal in developing BPGCs was to use conjugate formation with CD1d as a method for improving delivery of iNKT cell activators as potential agents for immunotherapy. Particularly in the case of cancer immunotherapy, many studies in

mouse models have highlighted the potential for iNKT cell activators to deliver striking anti-cancer effects.[4c] However, administration of α-GC as a free glycolipid has the potential for dose limiting toxicity, and also leads to the rapid development of a long-lived hyporesponsive state (anergy) that interferes with repeated treatments.[10, 21] Most likely, these issues contribute to the limited efficacy of free α-GC injections observed so far in early phase clinical trials for cancer in humans.[9, 22] Approaches to more precisely deliver α-GC to overcome these problems have been developed, including the administration of *ex vivo* glycolipid loaded antigen presenting cells or targeted soluble recombinant CD1d proteins.[4c, 8a, 8c, 8d] These approaches have shown improved outcomes in animal models, as well as in limited clinical studies in cancer patients[4c, 6b, 23]. However, the ability of α-GC to dissociate from these delivery vehicles after *in vivo* injection remains a suboptimal feature. The use of BPGCs to covalently lock the glycolipid onto CD1d in an active configuration, as shown in the current study, represents a method for improving these delivery methods, and achieving more focused and effective immunotherapy without the limitations or toxic effects of systemic α-GC injections.

Detailed analysis of the effects of variation in the length of the acyl chain spacer in the BPGCs revealed some interesting and potentially important findings. First, we noted that the **9** and **10** compounds had very low TCR affinity and low stimulatory activity when not covalently linked to CD1d (**Figs. 3f and 4a**), presumably because of their rapid dissociation. However, with conjugate formation these glycolipids showed improved affinity and enhanced iNKT cell stimulation. This suggests that the BPGCs with shorter acyl chains, even if released by some mechanism after conjugation, should

be less likely to induce unwanted effects such as iNKT cell anergy or systemic toxicity. Another notable finding was that **9**, which even after conjugation continued to show a lower TCR affinity than the other BPGCs, also gave a remarkably biased cytokine response (i.e., significant IFN-γ levels with no detectable IL-4 or other cytokines) when injected in the form of a conjugate with soluble CD1d protein (**Fig. 4b**). This type of "Th1-biased" cytokine response has been repeatedly associated in previous studies with analogues of α-GC that provide superior anti-tumor responses in mouse models.[24] Thus, through alterations in the length of the acyl chain spacer, it should be possible to tune the responses to BPGC conjugates in terms of affinity and biologic response to optimize desired therapeutic outcomes.

Our mapping of the site of covalent bond formation in **9** and **13** conjugates yielded the surprising finding of a single major conjugation site for both glycolipids. Another surprising aspect of this result was that the specific region of CD1d that was implicated was located near the base of the F' pocket, which in all CD1d-glycolipid complexes resolved by X-ray crystallography would be predicted to be in greater proximity to the sphingoid base than the acyl tail of the glycolipid (**Fig. 5c**).[2] However, this apparent switch in the orientation of the two lipid tails of the glycolipid can be readily accommodated in energetically favorable poses of the glycolipid in molecular models (**Fig. 6**). Thus, our findings suggest the possibility of greater flexibility in the lipid binding process of CD1d, which has only been occasionally hinted at in atomic level structure studies.[25] Further structural analyses of CD1d-BPGC conjugates should enable new types of analyses to expand our understanding of the unique process of

glycolipid antigen presentation, as well as opportunities for improving immunotherapies that target iNKT cells.

EXPERIMENTAL METHODS

Synthesis and compound characterization. Full experimental details of the synthesis and characterization of all compounds used in this study are provided in the **Supplementary Information**.

Mice. Female C57BL/6J (B6) mice 6–8 weeks old were obtained from Jackson Laboratories or Taconic and maintained in pathogen-free conditions. All experiments requiring mice were conducted in compliance with institutional guidelines and under an authorization delivered by the Institute of Animal Use and Biosafety Committee at Albert Einstein College of Medicine.

Cell lines, clones and hybridomas. HeLa cells transfected with human CD1d (HeLa.hCD1d) were cultured in DMEM supplemented with 10% FBS, and JAWS II cells were cultured in α-MEM supplemented with 10% FBS and 20 ng/ml murine GM-CSF. Mouse splenocytes were maintained in RPMI with 10% FBS. BMDCs were prepared as described earlier and cultured in RPMI supplemented with 10% FBS. Mouse iNKT hybridoma DN3A4-1.2 was maintained in complete RPMI medium containing 10% decomplemented serum.[26] Human iNKT clonal HDE3 was clonally expanded by stimulation with PHA-P in the presence of recombinant human IL-2 at 250 IU/ mL , recombinant human IL-7 at 10 ng/mL and allogeneic PBMCs (irradiated at 5000 rad),

and cultures were fed by addition of fresh medium containing IL-7 and IL-2 every 2-3 days.[8d, 15]

Recombinant CD1d proteins and monoclonal antibody L363. Single chain β2m-mouse CD1d-hexahistidine (MW ~57 kDa) and human CD1d-β2m-hexahistidine were purified from CHO cells stably transfected with respective genes.[27 15] Single chain mCD1d-β2m with a C-terminal single chain immunoglobulin Fv fusion (mCD1d-β2m-ScFv, MW ~78 kDa) was produced in transiently transfected HEK cells and purified as previously described.[8c, 8d] The ScFv moieties of these proteins were specific for the human tumor associated antigens CEA or C35, although their specific binding properties were not relevant to experiments in the current study.

To determine the affinity of mCD1d-BPGC tetramers for iNKT-cell TCRs, 1 x 10^4 DN3A4-1.2 cells were stained with a range of concentrations of tetramers loaded with the different glycolipids to form complexes. Soluble mCD1d proteins were prepared and biotinylated following published methods with minor modifications.[28] Glycolipids were prepared and loaded onto soluble mCD1d as described in the following section. In some cases, the loaded mCD1d complexes were converted to covalent conjugates by exposure to UV irradiation for 60 minutes in solution (400 mJoules/cm^2). Formation of tetramers, equilibrium binding of tetramers to NKT cells, and measurement of binding by flow cytometry has been previously described in detail.[15]

Monoclonal antibody L363, specific for mCD1d with bound α-GC C26:0 and other related forms of α-GC, has been previously described[15, 18, 29] The antibody was purified from supernatants of the cultured hybridoma line, and was biotinylated using sulfo-NHS

biotinylation kit or fluorescently tagged with Alexa Fluor 647 (Alexa 647 labelling kit, Thermo Fisher). Binding of fluorescently labeled L363 to cells was determined by flow cytometry using an LSR II cytometer (BD Biosciences) and FlowJo software.[15, 19]

CD1d loading and covalent crosslinking. Glycolipids dissolved in DMSO at 1 mg/ml were diluted to 100 μM concentration in appropriate volumes of PBS and PBS plus 0.1% Triton X-100 to yield final DMSO concentration of 8-10% and 0.05% Triton X-100. Glycolipids were added to CD1d at a molar ratio of 3:1 in PBS plus 0.05% Triton X-100 (for *in vitro* applications) or PBS + 0.05% Tween-20 (for *in vivo* applications), and incubated for 12-18 hours for complete loading of the complexes. Loaded complexes were transferred to ultra-low binding microtiter plate wells and cooled on ice. A fixed wavelength UV lamp (Schleicher & Schuell, RAD-FREE long wave UV lamp, λ = 365 nm) was placed directly over wells containing complexes for 1 hour on ice. Resulting conjugates were recovered from the wells and excess glycolipid and detergent was removed using detergent-removal columns (Pierce).

An azide-functionalized **13** was employed to determine the efficiency of covalent cross-linking. The azido-**13** conjugates and complexes were covalently coupled to dibenzenecyclooctyne tetramethylrhodamine (DBCO-TAMRA, Click Chemistry Tools) by Huisgen cycloaddition.[17] The ternary complex thus obtained was denatured using DTT and SDS and run on SDS-PAGE for detection. To determine stability of conjugates, the complexes and conjugates were coated onto high binding 96 well plates and washed with PBS-Tx 0.05% every 12 hours for three days to remove reversibly bound glycolipids. Plates were incubated at room temperature between washes.

For detection of mCD1d-glycolipid complexes and conjugates on glycolipid pulsed JAWS II cells, the cells were plated at 5 x 10^4 cells per well in microtiter plates and cultured with 100 nM of either α-GC C26:0 or **13** for 18 hours, followed by one wash with medium and then UV irradiation if indicated for 30 minutes (~400 mJ/cm^2). Samples of UV exposed and non-exposed cells were stained with mAb L363 coupled with AlexaFluor 647 and analyzed by flow cytometry using an LSR II flow cytometer (BD Biosciences) to determine surface bound glycolipid-CD1d complexes or conjugates (solid black bars), before and after incubation for two days to allow dissociation of glycolipids.

***In vitro* and *in vivo* iNKT-cell stimulation assays.** To determine the EC_{50} of BPGCs, BMDCs from C57BL/6 mice or human CD1d transfected HeLa cells (HeLa.hCD1d) were cultured in microtiter plate wells (5 x 10^4 BMDC and 1 x 10^4 HeLa.hCD1d per well), and pulsed with varying BPGC concentrations ranging between 50 μM and 0.01 nM in 100 μl of culture medium per well for 3 hours at 37 °C. Cells were washed once to remove unbound glycolipid. The iNKT hybridoma DN3A4-1.2 or human iNKT clone HDE3 were then added (5 x 10^4 cells per well in 0.2 ml medium), and the cultures were maintained for 12 – 18 hours at 37°C. Stimulation was determined by measuring supernatant levels of IL-2 for DN3A4 1.2 or IFN-γ for HDE3 by capture ELISA as described.[30] Cytokine response was plotted against dose and EC_{50} was calculated using the function log agonist against response in Prism software.

To determine the serum cytokine levels induced *in vivo* in mice by administration of free glycolipids, glycolipid-loaded mCD1d complexes and conjugates, or *ex vivo*

glycolipid loaded JAWS II cells, mice were bled at 2, 10 and 24 hours following i.v. injections and serum samples were prepared. For free glycolipids, mice (3-5 per group) received 4 nanomoles of α-GC C26:0, **9**, **13** or **15**. For comparison of *in vivo* activity of conjugates, complexes and free glycolipids, 30 μg/mouse of complexes or conjugates or equimolar amounts of free BPGCs (0.4 nanomoles) were injected into mice i.v. Serum cytokine levels were measured by capture ELISA.

Mass spectrometry and proteomic analyses. Tryptic and chymotryptic peptide digest mixtures of native mCD1d or conjugates were analysed either directly by on-line nano-liquid chromatography (nano-LC) electrospray (ES) MS and MS/MS, or subjected to *N*-linked glycan release by PNGase F followed by subdigestion with additional proteases prior to analysis. For detailed method see Supplemental Methods section.

Computational molecular modeling and docking studies. Binding modes of **9** and **13** glycolipids to mCD1d were predicted using the mCD1d/α-GC/TCR crystal structure with PDB entry code 3HE6, containing Vα14-Vβ8.2 iNKT TCR (mouse variable, human constant domains). The crystal structure was prepared using Protein Preparation Wizard tools (Schrödinger software package, version 2016-1). For detailed method see Supplemental Methods section.

Statistical analysis. Results are expressed as mean ± SEM. Statistical significance between the groups was determined with multiple t tests, one-way-ANOVA or two-way-

ANOVA tests with the Bonferroni correction as indicated. All statistical analyses were done using GraphPad Prism software.

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Competing financial interests

MZ is an employee of Vaccinex, Inc., which has proprietary interests in iNKT cell-based immunotherapeutics. SAP is a paid consultant of Vaccinex, Inc. The other authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper.

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