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Enhanced TCR Footprint by a Novel Glycolipid Increases NKT-Dependent Tumor Protection

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NKT cells, a unique type of regulatory T cells, respond to structurally diverse glycolipids presented by CD1d. Although it was previously thought that recognition of glycolipids such as α-galactosylceramide (α-GalCer) by the NKT cell TCR (NKTcR) obeys a key–lock principle, it is now clear this interaction is much more flexible. In this article, we report the structure–function analysis of a series of novel 6’’-OH analogs of α-GalCer with more potent antitumor characteristics. Surprisingly, one of the novel carbamate analogs, α-GalCer-6’’-(pyridin-4-yl)carbamate, formed novel interactions with the NKTcR. This interaction was associated with an extremely high level of Th1 polarization and superior antitumor responses. These data highlight the in vivo relevance of adding aromatic moieties to the 6’’-OH position of the sugar and additionally show that judiciously chosen linkers are a promising strategy to generate strong Th1-polarizing glycolipids through increased binding either to CD1d or to NKTcR. The Journal of Immunology, 2013, 191: 2916–2925.

Natural killer T cells are a subset of regulatory T cells that are involved in different pathological processes, ranging from autoimmunity to protection against tumors and bacterial infections (1). NKT cell activation results in cytotoxicity, proliferation, and also rapid cytokine production (within several hours), which subsequently activate several bystander immune cells (NK cells, dendritic cells, B cells, and so forth). They have the capacity to produce both Th1 and Th2 cytokines and to modulate production by bystander cells. As such, they have the ability to lead to Th biased responses under certain conditions. Although recently a lot of studies have been performed to unravel the mechanism for Th1/Th2 polarization (2–7), much remains to be uncovered.

These innate-like T cells recognize glycolipids in the context of CD1d, which is a monomorphic MHC I-like molecule that accommodates the lipid tails of the glycolipid in two hydrophobic pockets (A’ and F’) and presents the sugar head to the NKT cell TCR (NKTcR). The prototype invariant NKT cell (iNKT cell)–activating glycolipid is α-galactosylceramide (α-GalCer), whose chemical structure consists of a 26-carbon acyl chain and a phosphatidylcholine chain α-anomerically linked to galactose. Although initially iNKT cell research was mainly focused on this Ag, the list of novel glycolipids able to induce iNKT cell activation is continuously growing and includes very diverse bacterial Ags and endogenously expressed glycolipids, in addition to newly synthesized Ags (8, 9).

The iNKT cell TCR is semi-invariant, as it contains a conserved Vα14 chain in mice and Vα24 in humans that both rearrange with Jα18, whereas the Vβ-chain is more variable. However, only germline-encoded residues are important for the recognition of a glycolipid (10). Although the TCR plays an important role in initial recognition of the CD1d–glycolipid complex, the strength of a Th1-polarized iNKT cell–dependent activation seems to be more determined by the stability of the CD1d–glycolipid complex. Previously we showed that α-GalCer-6’’-(1-naphthyl)urea (NU-α-GalCer) induces a structural change within the A’ roof of CD1d, to which it binds with its hydrophobic 6’’-naphthylurea group, leading to the so-called third anchor model (7). However, extra binding strength of a glycolipid can also be achieved through

S.A. performed and designed mouse experiments; K.V. performed human iNKT cell experiments; M.N. performed surface plasmon resonance and crystallographic experiments; N.P. and S.V.C. synthesized the glycolipids; J.W. prepared recombinant proteins; S.A., M.N., D.M.Z., and D.E. designed experiments and analyzed the data; and S.A., M.N., D.M.Z., and D.E. wrote the manuscript.

The structure factors and coordinates presented in this article have been submitted to Protein Data Bank (www.rcsb.org) under accession numbers 4IRJ (4ClPhC-α-GalCer) and 4IRS (PyrC-α-GalCer).

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alterations of the lipid tails (11, 12). The altered sphingosine chain of a plakoside analog was shown to increase the contact surface area with CD1d within the F'-pocket (6). In addition, it was shown that several acyl chain–altered glycolipids can induce superior anticancer effects compared with the effects of α-GaLCer, and this was also linked to increased CD1d avidity (13). OCH, in contrast, has a shorter sphingosine chain (C9 instead of C18) and is therefore not able to induce the formation of the F’ roof in CD1d, which affects the recognition by the NKTRC and thus its antigenicity (5, 14). Last, but not least, crystallographic analysis of bacterial glycosyl-diacylglycerol lipids, as well as Igβ, which is a β-anomeric trihexose containing sphingolipid self-antigen, demonstrated that the TCR was able to “bulldoze” the three sugar groups over the CD1d surface, thus allowing the TCR to bind to the CD1d–glycolipid complex with its conserved footprint (15–18). For Igβ3, this mechanism induces the last “anchor” sugar to bind to CD1d; however, this doesn’t appear to happen to Gb3, which only differs by an altered linkage of the last sugar, because the position of the terminal sugar of Gb3 likely does not favor the formation of this additional anchor to CD1d (17). However, a similar, yet energetically unfavorable linker can be enforced in Gb3 through mutation of the TCR to reach sufficiently high auto-reactivity for CD1d (18). Besides its role in enhancing Th1 polarization, the stability of the CD1d–glycolipid complex also seems to determine the antigenicity of certain glycolipids for iNKT cells. It was demonstrated for α-C-GaLCer, a well-known Th1-polarizing glycolipid with antitumor properties, that its complex with CD1d had a much longer half-life in vivo than the corresponding CD1d–α-GaLCer complexes (5). Therefore, structural features that enhance the binding stability between a glycolipid and CD1d seem to enhance both its antigenicity and its Th1-polarizing properties.

Our previous data suggested that the formation of an extra anchor between a glycolipid and CD1d confers stronger anti-tumoral responses in vivo. However, the Th1-polarizing strength seemed to be critically dependent on the nature and length of the linker between C-6’ of the galactose and the aromatic group. BrNH-GSL-1’, an analog characterized by an aromatic moiety that is located one atom closer to the galactose ring, was shown to exhibit weaker TCR affinity and decreased antigenicity. Despite the fact that this glycolipid also carries an amide linker, similar to the urea linker of NU-α-GaLCer, it does not form an additional anchor with CD1d, likely as a result of the different linker length (7). To explore the structural modifications required for the formation of the third anchor, three novel 6’-OH altered glycolipids were synthesized containing a carbamate linker, which has the same length as NU-α-GaLCer but increased flexibility. They are able to induce superior Th1 responses in mice and also activate human iNKT cells. In contrast to most Th1-polarizing analogs, the Th1 polarization potency did not only on the induction of a different Th1/Th2 polarization balance but also on a much higher induction of Th1 cytokines, compared with α-GaLCer, which was independent of the mode of administration. We show that in analogy to NU-α-GaLCer these carbamates display an increased binding stability for CD1d, compared with α-GaLCer, further emphasizing the relevance of this model in iNKT cell–driven responses. Furthermore, we demonstrate that one glycolipid exhibits better antitumor activity than NU-α-GaLCer. Surprisingly, structural characterization of two of the potent carbamate glycolipids revealed that none of the aromatic groups form the third anchor to the degree observed for NU-α-GaLCer. Instead, the most potent Th1-skewing glycolipid, α-GaLCer-6’-(pyridin-4-yl)carbamate (PyrC-α-GaLCer), does form an additional “anchor” with the TCR, leading to the highest observed TCR binding affinity of all studied α-GaLCer analogs to date, but equal to that of α-GaLCer itself.

Materials and Methods

Synthetic glycolipids

Glycolipids were synthesized in the Laboratory of Medicinal Chemistry (19). α-C-GaLCer was kindly provided by M. Tsuji (Aaron Diamond AIDS Research Center, New York, NY) and the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Lysophospholipid glycolipids were dissolved in pure DMSO (Sigma–Aldrich) at 10 mg/ml concentration and stored at −20°C. Glycolipids were further solubilized by adding PBS (Invitrogen) or vehicle (96 mg/ml sucrose, 10 mg/ml sodium deoxycholate, 0.05% Tween 20), warming to 80°C for 20 min, and sonication for 10 min.

Cell lines

The murine (m) iNKT hybridoma N38-2C12 (Va14Vß8.2b) was provided by L. Brossay (20) (Brown University, Providence, RI). Cells were cultured in DMEM (Sigma–Aldrich) supplemented with 10% FCS (Invitrogen), 1% glutamine (Sigma–Aldrich), 1% penicillin streptomycin (Sigma–Aldrich), and 2-ME (Sigma–Aldrich) (called cDMEM hereafter). B16 melanoma cells were cultured in advanced RPMI 1640 (Sigma–Aldrich) supplemented with 10% FCS (Invitrogen), 1% glutamine (Sigma–Aldrich), and 1% penicillin streptomycin (Sigma–Aldrich). They were harvested using cell dissociation buffer, which was washed away twice, first using the medium and then with PBS. Next, 400,000 cells were i.v. injected into the tail vein within 30 min after harvest.

Isolation and expansion of bone marrow dendritic cells

Bone marrow dendritic cells (BMDCs) were isolated from the mouse bone marrow, as described previously (21).

Mice

C57BL/6 and CD45.1 mice were bred in house (in accordance with the general recommendations for animal breeding and housing) or purchased from the Harlan Laboratory; Jα18-knockout mice on the C57BL/6 background were kindly provided by M. Taniguchi (22) (RIKEN, Tsurumi, Yokohama, Japan). Experiments were conducted according to the guide-lines of the Ethical Committee of Laboratory Animals Welfare of Ghent University. Mice used for experiments were between 5 and 12 wk old.

In vivo tumor model

Within 30 min after harvesting, a dose of 2 × 106 or 4 × 106 B16 cells was inoculated i.v. (tail vein). Mice were killed 14 d later, the lungs were removed, and surface metastases were counted with the aid of a dissecting microscope.

In vitro and in vivo activation of iNKT cells

For in vitro stimulation, murine iNKT hybridoma cells at 5 × 105 cells per well in 96-well plates were stimulated with the 103 cells per well glycolipid-pulsed BMDCs in cDMEM for 4, 16, or 24 h at 37°C, and levels of IL-2 secretion were determined by ELISA.

For in vivo activation of iNKT cells, C57BL/6 mice were either i.p. injected with 5 μg glycolipid (dissolved in PBS) or i.v. with 6 × 1010 or 1 × 1010 glycolipid-pulsed BMDCs.

Isolation of human PBMCs and iNKT cells

Human iNKT cells from healthy adult individuals were sorted and expanded as described previously (7). PBMCs were isolated by means of density centrifugation, incubated overnight in the presence of indicated glycolipids (100 ng/ml), washed, and irradiated (40 Gy). Subsequently, 5 × 104 iNKT cells were stimulated with 103 glycolipid-pulsed autologous PBMCs in RPMI 1640 media supplemented with 10% human AB serum (Lonza), 1% sodium pyruvate, 1% nonessential amino acids, and 1% penicillin/streptomycin (all from Invitrogen). Supernatants were collected after 24 h of culture, and cytokine levels were determined by means of Cytometric Bead Arrays (CBA) following the manufacturer’s instructions (BD Biosciences).

Isolation of murine lymphocytes

Spleen cells were isolated as previously described (23). Lymphocytes were isolated at the interface and washed, depleted with an anti-CD3 kit (Miltenyi Biotech), and resuspended in staining buffer containing a saturating amount of anti-Fcy receptor type II/III monoclonal Abs (Miltenyi Biotech). Hereafter, cells were stained with fluorochrome-conjugated mAbs (all from eBioscience) directed against the described Ags. Live cells (exclusion with DAPI) were acquired on a FACSCanto (BD Biosciences) flow cytometer and analyzed using FlowJo (TreeStar) software.
**Surface plasmon resonance experiments**

Glycolipids were dissolved in DMSO at 1 mg/ml and stored at ~20°C. Surface plasmon resonance studies were conducted using a Biacore 3000, as reported previously (24). Briefly, biotinylated biA-tagged mCD1d protein was loaded overnight with 6 times molar excess of glycolipids, as previously reported (16), and 500–600 resonance units of mCD1d–glycolipid complexes were captured on a streptavidin sensor chip surface (GE Healthcare). TCR protein was diluted in detergent-free running buffer (10 mM HEPES, 150 mM NaCl, and 3 mM EDTA, pH 7.4). The TCR was injected in serial dilutions (0, 0.0156–2 μM) for 1.5–3 min at 30 μl/min to measure the association phase while dissociation was continued for 45 min at 25°C. A reference surface containing “empty” CD1d was generated in a flow channel of one of the streptavidin–sensor chips, and its TCR binding response was subtracted from the other sensorgrams before calculating binding kinetics, using a simple Langmuir 1:1 model in BIAevaluation software version 4.1. Experiments were performed three times, each using a different TCR preparation.

**Crytallographic and structure determination**

The ternary mCD1d–glycolipid–TCR complexes using the lipids α-GalCer, 6'-4-chlorophenyl)carbamate (4ClPhC-) and PyrC-α-GalCer were synthesized containing a carbamate-based linker instead of the α-OH-based, instead of a 6'-N-based derivatization, which in this context is also significantly higher than α-GalCer (Fig. 2). Strikingly, all carbamate-linked glycolipids induced significantly higher IFN-γ levels than did NU-α-GalCer and α-GalCer (Fig. 1). In this setting, NU-α-GalCer induced comparable or slightly lower IFN-γ production compared with α-GalCer. IFN-γ production in response to glycolipid-dependent iNKT cell activation is known to be dependent on IL-12 (34–36), which was therefore analyzed. As expected, IL-12 production was also significantly higher for the carbamate-based glycolipids compared with α-GalCer. However, only PyrC-α-GalCer was capable of inducing significantly higher IL-12 levels than NU-α-GalCer. Additional pharmacokinetic analyses confirmed that PyrC had a superior Th1 profile (data not shown). Administration of these novel glycolipids to Jx18+ mice, which lack iNKT cells, did not induce any cytokine production, thereby excluding nonspecific effects (Supplemental Fig. 1).

Next we examined whether the glycolipid-pulsed BMDCs behave similarly in vivo. For cytokine analysis we bled the mice at several time points after injection. We focused on IFN-γ and IL-12 secretion. Again at 16 h, both carbamates induced a significantly higher IFN-γ secretion than did NU-α-GalCer (which in this context is also significantly higher than α-GalCer) (Fig. 2). Strikingly, for PyrC-α-GalCer the IFN-γ level increased up to 24 h after injection, in contrast to 4ClPhC-α-GalCer and NU-α-GalCer. Similarly, IL-12 secretion was very high with PyrC-α-GalCer and even after 24 h markedly higher than for NU-α-GalCer. Furthermore, PyrC-α-GalCer and the other 6'-OH-based, instead of a 6'-N-based derivatization, were able to induce Th1-biased cytokine secretion (more IFN-γ and IL-12 and less IL-4 and IL-13 compared with α-GalCer) in cultures of purified human iNKT cells (Fig. 3 and data not shown). A similar trend was seen with human PBMCs (data not shown), highlighting the conserved nature of the Th polarization.

6'-OH analogs modulate the costimulatory landscape

Expression of costimulatory markers at the cell surface is linked to cytokine polarization because this determines the degree of activation of bystander cells such as NK cells, whose IFN-γ production is responsible for the Th1 polarized cytokine profile (37). For α-C-GalCer, it has been shown that expression of CD40 is essential for IL-12 and subsequent NK cell–dependent IFN-γ production (3). In addition, OX40L upregulation by DCs has been found to be important for α-GalCer–dependent tumor killing (38). In this article, we show that PyrC-α-GalCer, which induces the highest levels of IL-12, induces early CD40 and OX40L upregulation on spleen

**Carbamates are strong Th1 polarizers in vivo**

To assay the antigenicity of these carbamate analogs and their ability to induce Th1 skewing, mice were bled at 16 h after i.p. glycolipid exposure because this is known to afford peak levels of IFN-γ, the hallmark Th1 cytokine. Strikingly, all carbamate-linked glycolipids induced significantly higher IFN-γ levels than did NU-α-GalCer and α-GalCer (Fig. 1). We show that PyrC-α-GalCer is capable of inducing significantly higher IL-12 levels than NU-α-GalCer. Additional pharmacokinetic analyses confirmed that PyrC had a superior Th1 profile (data not shown). Administration of these novel glycolipids to Jx18+ mice, which lack iNKT cells, did not induce any cytokine production, thereby excluding nonspecific effects (Supplemental Fig. 1).

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**Statistical analysis**

The statistical test used throughout this study was the Kruskal–Wallis test with the Dunn multiple comparison test or Mann–Whitney U test (unpaired, two-sided) unless otherwise stated. Data were analyzed using Excel (Microsoft) and GraphPad Prism 5.

**Results**

Previously, we have shown that α-GalCer analogs with alterations at the 6'-OH position of the sugar head group are potent inducers of
DCs (CD11c CD11b double positive), both quantitatively and qualitatively (Supplemental Fig. 2). ICOSL expression has been related to production of Th2 cytokines by marginal zone B cells (39) and was shown to be important for cytokine production and survival of CD4-positive iNKT cells (40). The shifts in ICOSL expression were overall minimal, so the exact relationship between ICOSL expression and the superior Th1 polarization by PyrC-α-GalCer remains to be determined. Glycolipid stimulation of iNKT cells leads to CD28 expression, and induces CD80 and CD86 expression at the dendritic cell surface, which is required for induction of IL-12 by dendritic cells (41). This idea was confirmed in this study, as all glycolipids, including α-GalCer, induced upregulation of both CD80 and CD86.

**TCR affinity and stability of the CD1d–glycolipid complex**

The iNKT cell polarization is a matter of debate, and it has been shown that uptake by different cells can also affect the outcome (42, 43). To avoid these host-dependent parameters, we set up a simple

**FIGURE 1.** Th1–Th2 profile of novel glycolipids: serum cytokine levels at 16 h after injection of 5 μg glycolipid (i.p.). Graphs indicate the mean with SEM of ≥16 mice. Data are pooled from two independent experiments. (A) IFN-γ levels. (B) IL-12 levels. Kruskal–Wallis and two-tailed Mann–Whitney U tests were used: *p < 0.05 and **p < 0.01 as compared to α-GalCer. †p < 0.05, ‡p < 0.01, and §§p < 0.001 as compared to NU-α-GalCer.

**FIGURE 2.** Th1–Th2 profile of novel glycolipids loaded on BMDCs (20 h, 100 ng/ml): serum cytokine levels at different time points after injection of glycolipid-pulsed BMDCs. Graphs indicate the mean with SEM of 16 mice. Data are pooled from two independent experiments. (A) After injection of 600,000 glycolipid-pulsed BMDCs, IFN-γ levels of 4CIPhC and PyrC-α-GalCer are both, at 16 and 24 h, significantly higher than those of NU-α-GalCer. NU-α-GalCer also induces IFN-γ levels that are significantly higher compared with those of α-GalCer, at both 16 and 24 h after injection. (B) After injection of 600,000 glycolipid-pulsed BMDCs, IL-12 levels of PyrC-α-GalCer are both, at 16 and 24 h, significantly higher compared with those of NU-α-GalCer. NU-α-GalCer and 4CIPhC also induce IFN-γ levels that are significantly higher than those of α-GalCer at 16 h after injection. (C and D) Both IFN-γ and IL-12 levels are significantly higher 16 h after injection of only 10,000 PyrC-pulsed BMDCs, compared with those of NU-α-GalCer and α-GalCer. For (A)–(D) Kruskal–Wallis and two-tailed Mann–Whitney U tests were used. **p < 0.01 and ***p < 0.001 as compared to α-GalCer. †p < 0.05, ‡p < 0.01, and §§p < 0.001 as compared to NU-α-GalCer.
in vitro model consisting of cocultures of glycolipid-pulsed BMDCs and an iNKT cell hybridoma [i.e. 2C12 containing a Vβ8.2 TCR (44)]. IL-2 production is used as a read-out for TCR affinity for the whole CD1d–glycolipid complex. In this case, the carbamates NU-a-GalCer and a-C-GalCer induce higher IL-2 levels (Fig. 4). Additional dose–response assays verified the superior iNKT hybridoma cell responses toward PyrC versus a-GalCer–loaded BMDCs (data not shown). To confirm this, we also measured intracellular IL-2 production in 2C12 cells 4 h after coculture, for which a similar result was obtained (Fig. 4). It was reported that BMDCs can also produce IL-2 (45); however, intracellular IL-2 staining of BMDCs was negative (data not shown). Because IL-2 production is a downstream event of TCR signaling, we analyzed TCR β expression. In vivo it is well known that iNKT cells internalize their TCR upon Ag recognition (46). Fig. 4 suggests that TCR internalization also occurs in vitro and correlates well with the intracellular IL-2 production.

However, IL-2 production and TCR triggering are the result of both molecular TCR affinity and CD1d stability. Therefore, we assessed the equilibrium binding constants (KD) of the TCR toward the different glycolipids presented by CD1d, using surface plasmon resonance. Overall, the data show similar affinities for the tested glycolipids PyrC-α-GalCer (KD = 25 nM), α-GalCer (KD = 26 nM), NC-α-GalCer ([α-GalCer-6″-(1-naphthyl)carbamate]; KD = 37 nM), and 4ClPhC-α-GalCer (KD = 49.3 nM) (Table I). Thus, in contrast to BnNH-GSL-1”, the glycolipid presentation by CD1d and/or the glycolipid in teraction with the TCR is not significantly affected by the carbamate-linked aromatic groups. Although 4ClPhC-α-GalCer shows a 2-fold reduced binding affinity (49 nM versus 25 nM), the structurally related PyrC-α-GalCer has a binding affinity equal to that of α-GalCer (25 nM). Therefore, the slightly different 6′′-OH modifications also translate into only marginally different binding affinities for the TCR, suggesting similar binding chemistries with the TCR. In addition, we investigated the role of CD1d–glycolipid stability, which has recently been shown to be important for antigenic iNKT responses (6, 7, 13). We used a cellular assay to determine the binding stability of the novel glycolipids. BMDCs were loaded with 100 ng/ml glycolipid for 20 h. After removal of the free glycolipid, cells were left in an appropriate medium for several time intervals (ranging from 4 h to 48 h). Dissociated glycolipid was removed, and coculture with 2C12 cells was initiated. IL-2 production in the medium was used as a surrogate marker for the remaining CD1d with glycolipid. The strong Th1-polarizing a-C-GalCer, charac-
terized by a higher binding stability to CD1d, was also included in this assay. IL-2 levels were normalized to the values of 4 h after washoff, to exclude the effect of TCR affinity. Fig. 5B clearly shows that all 6'-OH analogs and α-C-GalCer behave very similarly and have a much slower decay compared with α-GalCer. We conclude that all tested Th1 analogs have a similar stability with CD1d in vitro, which is much higher than for the CD1d-α-GalCer complex.

**Crystal structure of the mCD1d-PyrC-α-GalCer–TCR and mCD1d-4ClPhC-α-GalCer–TCR ternary complexes**

We previously reported the structural details of how another potent Th1-skewing glycolipid, NU-α-GalCer, interacts with CD1d and the TCR of iNKT cells (7). In that case, the NU-group faces down into the CD1d binding groove to form an additional anchor with CD1d, leading to increased CD1d stability. We therefore sought to determine whether the glycolipids PyrC-α-GalCer and 4ClPhC-α-GalCer follow a similar binding mode, because their 6'-OH modifications are connected to the galactose of α-GalCer via a more flexible carbamate linker (Fig. 1). We determined the crystal structures of the ternary complexes CD1d-PyrC-α-GalCer–TCR and CD1d-4ClPhC-α-GalCer–TCR to resolutions of 2.8 Å and 3 Å, respectively (Supplemental Table I, Fig. 6). Surprisingly, although the binding of both glycolipids is highly similar to that of α-GalCer, neither of the aromatic substitutions of PyrC-α-GalCer and 4ClPhC-α-GalCer insert down into the CD1d binding groove, as has been demonstrated for NU-α-GalCer (Fig. 6). Instead, both 6'-OH aromatic substitutions are presented differently above the A' pocket of CD1d. Although 4ClPhC-α-GalCer does not induce a structural change in the A' roof of CD1d, it interacts more intimately with CD1d. In contrast, PyrC-α-GalCer is slightly elevated and its pyridine group contacts the TCR (Fig. 6B, 6C). Similar to α-GalCer, the galactose moiety of both glycolipids forms H-bonds with the TCR through the 2''- and 3''-OH groups, whereas the 4''-OH group loses this contact. Of interest, it has been shown that the TCR contact with the 4''-OH group is the key determinant in TCR interaction (14). However, both PyrC-α-GalCer and 4ClPhC-α-GalCer lost the H-bond [while still making a van der Waals (VDW) contact with 3.5–3.8 Å distance] and still have similar binding affinities compared with α-GalCer, suggesting that the 6'-OH modifications compensate in part for the loss of the 4''-OH H-bond with the TCR. Most surprisingly, major VDW interactions were observed between the pyridine of PyrC-α-GalCer and the Gln52 of the TCR (Fig. 6C). The pyridine makes intimate contacts with Gln52 (distance 3.3–3.5 Å) compared with 6.4–6.9 Å for the 4Cl-phenyl group (4ClPhC-α-GalCer) and 8.0–12.9 Å for the naphthyl group (NU-α-GalCer) (Fig. 7). As a result, the TCR exhibits many more interactions with PyrC-α-GalCer than with 4ClPhC-α-GalCer or NU-α-GalCer (7), leading to the observed high-affinity TCR binding (Table I). Even though the pyridine ring forms extra contacts with Gln52 of the TCR, the binding affinity does not exceed the binding affinity of mTCR to α-GalCer-CD1d, likely because it lacks the important H-bond between the 4''-OH of galactose and Asn39 of the TCR. Interestingly, the additional glycolipid contact with TCR residue Gln52 has previously not been seen in any other structure. Therefore, our data provide a structural framework for the design of novel α-GalCer analogs that target Gln52 to increase TCR contacts.

**Binding of 4ClPhC-α-GalCer and PyrC-α-GalCer to CD1d equals that of α-GalCer. All the H-bond interactions between the 2''-OH (with Asp55) and 3''-OH (with Asp53) of the glycolipid galactose and the 3'-OH (with Asp30), 4'-OH (with Asp30) of the glycolipid ceramide backbone with CD1d residues (outlined) are conserved (Fig. 6D). Carbamate-linked aromatic groups of 4ClPhC-α-GalCer and PyrC-α-GalCer, however, do not form the extra H-bond interactions between the carboxyl oxygen of the urea linker that connects the galactose and the naphthyl moieties with Thr599 of CD1d, as shown in urea-linked NU-α-GalCer. Thus, the carbamate linker results in a more flexible presentation of the substituents as well as a less tight interaction of the linker itself with CD1d, explaining the slightly less well ordered electron density for the glycolipid 6'-OH modification, versus NU-α-GalCer (Fig. 6B) (7). Analysis of the buried surface areas (a measure of the extent of how two molecules contact each other) between the

### Table I. TCR binding kinetics

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>$K_D$ (nM)</th>
<th>$K_{on}$ (1/Ms)</th>
<th>$K_{off}$ (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-GalCer</td>
<td>26.45 ± 4.6</td>
<td>5.06 ± 0.6610⁴</td>
<td>1.34 ± 0.0910⁻³</td>
</tr>
<tr>
<td>4ClPhC-α-GalCer</td>
<td>49.3 ± 0.3</td>
<td>5.27 ± 1.4710⁴</td>
<td>2.62 ± 0.7210⁻³</td>
</tr>
<tr>
<td>NC-α-GalCer</td>
<td>37.1 ± 14</td>
<td>3.83 ± 0.5310⁴</td>
<td>1.5 ± 0.07410⁻³</td>
</tr>
<tr>
<td>PyrC-α-GalCer</td>
<td>25.0 ± 3</td>
<td>6.61 ± 1.2110⁴</td>
<td>1.61 ± 0.0110⁻³</td>
</tr>
</tbody>
</table>

**FIGURE 5.** Analysis of stability of the glycolipid-CD1d complex. (A) BMDCs were first loaded for 20 h with 100 ng/ml glycolipid. Thereafter, the glycolipid was washed away, cells were left in appropriate medium for several time intervals (ranging from 4 h to 48 h; see x-axis). Medium with glycolipid that might have come off CD1d was washed away and coculture with 2C12 cells (iNKT cell hybridoma) was done. IL-2 production in the medium was used as a surrogate marker for glycolipid-loaded CD1d complexes. (B) Data from (A) were normalized to the 4-h time point. Data represent stability of each glycolipid toward CD1d. Data are shown as mean with SEM ($n = 6$). One representative of two independent experiments.
glycolipids and CD1d indicates that 4ClPhC-α-GalCer binds as extensively to CD1d as NU-α-GalCer (1124 versus 1146 Å²), correlating with their enhanced CD1d stability (Fig. 5B), whereas PyrC-α-GalCer (1045 Å²) and α-GalCer (1027 Å²) interact less extensively with CD1d (Table II). PyrC-α-GalCer, however, interacts more with the TCR α-chain (199.2 Å² versus 137.1–155.7 Å²) and also shows an increased stability when bound to CD1d (Fig. 5B). The increased contacts of 4ClPhC-α-GalCer are mostly the result of novel or increased VDW interactions with CD1d residues, including Met⁶⁹, Met¹⁶², and, more importantly, Thr¹⁵⁹ (Supplemental Table II). Glycolipid contacts with Thr¹⁵⁹ are not formed when α-GalCer or PyrC-α-GalCer binds to CD1d.

As a result, we observe two different glycolipid binding modes compared with α-GalCer: glycolipids that form increased contacts with CD1d (NU-α-GalCer and 4ClPhC-α-GalCer) and glycolipids that form increased contacts with the TCR (PyrC-α-GalCer).

Comparison of the presentation of both carbamate-linked, as well as urea-linked aromatic substitutions reveals the paucity of binding orientations that are adopted by the different chemical groups (Fig. 7). From forming a third anchor inside CD1d (NU-α-GalCer and 4ClPhC-α-GalCer) and glycolipids that form increased contacts with the TCR (PyrC-α-GalCer), the aromatic groups bridge ~11 Å between the CD1d and TCR interface, with the capacity to induce structural changes within CD1d, depending on the composition of the linker and aromatic substitution.
Carbamate-pulsed BMDCs confer strong antimetastatic potential

To determine if the new glycolipids can also mimic the antimetastatic activity of NU-α-GalCer, we examined their impact in the B16 lung melanoma model. Even though NU-α-GalCer was significantly stronger than α-GalCer, PyrC-α-GalCer was still superior in preventing tumor metastasis in the B16 lung model (Fig. 8). As little as 10⁴ pulsed BMDCs were enough to exert this superior in preventing tumor metastasis in the B16 lung model. Crystallographic results with the high dose and the antimetastatic results, PyrC-α-GalCer caused significantly higher IL-12 and IFN-γ production (Fig. 2C, 2D).

Discussion

We report the results of a novel group of 6′′-OH analogs with superior Th1-polarizing potential. Previously described Th1-polarizing glycolipids mostly exhibited weaker antigenicity, and their Th1 polarization mainly stemmed from a much weaker Th2 cytokine production (3, 13, 47). In contrast, the carbamate analogs described in this article display a significantly stronger Th1 profile owing to significantly higher IFN-γ and IL-12 production, compared with that of α-GalCer. Strikingly, this strong response was independent of the administration mode (soluble or loaded on BMDCs). In addition, all tested 6′′-OH analogs induced a superior antitumor response compared with α-GalCer. PyrC-α-GalCer, the glycolipid that elicited the strongest cytokine response, was also most potent in preventing lung metastasis in a tumor model. Crystallographic analysis from the trimolecular complex shows that this carbamate analog forms increased and novel contacts with the TCR.

Because several diseases are characterized by an unbalanced cytokine response, skewing the iNKT Th1/Th2 response can be an interesting treatment option. Hence, understanding how glycolipid-dependent iNKT cell activation can result in these contrasting cytokine profiles has been addressed by several groups and is important for the design of novel glycolipids with therapeutic properties. Different mechanisms both at the molecular and at the cellular level have been proposed. Recently, several reports have emphasized the stability of the CD1d–glycolipid complex to severely affect both the Th1-polarizing potency and the antigenicity of a glycolipid. This has been shown for structurally diverse glycolipids, each displaying a different mechanism for the increased stability. First, alteration of the lipid chains by the introduction of aromatic or cyclopropane groups enhanced CD1d stability and strengthened Th1 responses (6, 13, 48). Second, Tynziik et al. (6) showed that introduction of cyclopropane groups in both acyl and sphingosine chains enhanced CD1d stability. Third, previous research from our own group showed that the addition of extra linkers to the galactose could make additional contacts to CD1d, which resulted in increased CD1d stability and significantly more potent antitumor properties along with a Th1-polarized response. Fourth, although α-C-GalCer is a weaker Ag, it was shown that its complex with CD1d had a prolonged half-life (5), probably because of its resistance toward enzymatic degradation by glycosidases. Our results reported in this article show that all novel carbamate analogs form complexes with the cell surface CD1d that are at least as stable as those with α-C-GalCer and NU-α-GalCer. This finding confirms the importance of the stability of the CD1d–glycolipid complex in

![FIGURE 8. Tumor suppression by iNKT cell stimulation in a B16 melanoma lung metastasis model. (A) When 10,000 BMDCs loaded with glycolipid were injected, all 6′′-OH altered glycolipids are able to reduce the quantity of lung nodules significantly more than α-GalCer. In addition, PyrC-α-GalCer is able to reduce the number of nodules to an even significantly greater degree. Each dot represents an individual mouse with at least eight mice per group. Data are representative of two independent experiments. Error bars express the mean, and Kruskal–Wallis test and one-sided Student t test were used for statistical analysis. (B) Lungs used for analysis in (A). Each lung is representative of the specific glycolipid; the lung with the number of nodules that is the closest to the mean (number in the left corner) is shown here.]

<table>
<thead>
<tr>
<th>Contact Surfaces</th>
<th>α-GalCer</th>
<th>NU-α-GalCer</th>
<th>4CIPhC-α-GalCer</th>
<th>PyrC-α-GalCer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1d–ligand</td>
<td>1027</td>
<td>1146</td>
<td>1124</td>
<td>1045</td>
</tr>
<tr>
<td>TCRα–ligand</td>
<td>137.1</td>
<td>146.2</td>
<td>155.7</td>
<td>199.2</td>
</tr>
<tr>
<td>TCRα–CD1d</td>
<td>433.4</td>
<td>427.9</td>
<td>436.3</td>
<td>434.9</td>
</tr>
<tr>
<td>TCRβ–CD1d</td>
<td>192.6</td>
<td>324.0</td>
<td>298.9</td>
<td>319.3</td>
</tr>
<tr>
<td>TCRαβ–CD1d</td>
<td>626</td>
<td>751.9</td>
<td>735.2</td>
<td>754.2</td>
</tr>
</tbody>
</table>

CD1d–Ligand–TCR Complex

α-GalCer

NU-α-GalCer

4CIPhC-α-GalCer

PyrC-α-GalCer

Table II. Buried surface areas between TCR–CD1d and glycolipid (in Å³)
the observed Th1 bias. Despite the apparent lateral binding of PyrC-α-GalCer above the A’ roof of CD1d, similar contacts are formed with CD1d when compared with the binding of NU-α-GalCer, which binds in between the α1–α2 helix. Moreover, the trimolecular structure with PyrC-α-GalCer shows novel additional contacts between the TCR (residue Gin25) and the pyridine, which have previously not been seen in any other structure. Therefore, we believe that our data provide a structural framework for the design of novel α-GalCer analogs that target Gin25 to increase TCR contacts. This enlargement of the TCR footprint may contribute to an enhanced stability of the trimolecular complex and thus higher cytokine production.

The cytokine production of iNKT cells themselves cannot be polarized (49). However, polarization of the response depends mainly on the degree of activation of bystander cells, such as NK cells. It is believed that the IFN-γ initially produced by the iNKT cell itself induces APCs to produce IL-12 and IL-18 and subsequently drive NK cell–dependent IFN-γ production (34, 50). Upregulation of costimulatory molecules is crucial for the onset of this IL-12 production (36, 41, 51) and plays an important role in the polarization of the iNKT cell response. Increased upregulation of costimulatory molecules might be linked to increased stability of the trimolecular complex because a more stable immunologocal synapse might have more time for recruitment of these molecules. However, at present this hypothesis remains to be proven. The superior Th1 effect of α-C-GalCer has been attributed to superior induction of CD40 and CD40L ligation, compared with that of α-GalCer (3). Our results indicate markedly increased IL-12 production, along with an enhanced upregulation of CD40 and OX40L, by PyrC-α-GalCer–pulsed BMDCs compared with NU-α-GalCer– and α-GalCer–pulsed BMDCs, confirming that costimulation has an important role in expanding Th1 signalization. Two other costimulatory molecules, CD80 and CD86, were already almost maximally upregulated with α-GalCer. Therefore, no additional increase with 6′-OH–altered glycolipids could be observed. This finding is in line with the study by Fujii et al. (37), who found that CD80 and CD86 expression is less essential for adjuvant characteristics of glycolipids compared with CD40. Th2-polarizing glycolipids are generally more hydrophilic and thus characterized by shorter lipid tails and/or insertion of unsaturated bonds into the lipid tails (2, 4, 52). These glycolipids are, rather, loaded onto CD1d at the cell surface; moreover, these hydrophilic glycolipids are rapidly displaced from CD1d in the lysosome (4). In contrast, facilitated loading by lipid transfer proteins in the lysosome is essential for Th1 and mixed Th1/Th2 glycolipids. In addition, cell surface loading of CD1d has been associated with presentation of the CD1d–glycolipid complex outside of lipid rafts. This feature is postulated to confer a different immunological synapse and hence results, for example, in exclusion of certain costimulatory markers and in less IFN-γ production by bystander NK cells (2). Although we did not specifically test the involvement of lipid rafts or lysosomal loading, the fact that both strategies (loading onto BMDCs and soluble injection) resulted in a comparable Th1 polarization excludes an important role for differential loading of glycolipids. CD1d requires its cytoplasmic tail for recycling to the lysosome, so APCs expressing the tail-deleted form of CD1d are not able to load lysosomal glycolipids. We previously found that NU-α-GalCer and α-GalCer displayed similar results when presented by APCs with the tail deleted form of CD1d, indicating that 6′-derived glycolipids have a requirement for lysosomal loading similar to that of α-GalCer. Additional insertion of aromatic groups at the carbohydrate head moiety will probably not affect stability of the CD1d–glycolipid complex in the acid milieu of the lysosome because this is mainly due to lipid backbone alterations, which makes this an unlikely explanation for our results.

In conclusion, we show that structural optimization may afford analogs that combine increased Th1 potency (both in mice and men) with significantly stronger anticancer responses. In addition, PyrC-α-GalCer administration induces a 10-fold increase in IL-12 levels and is accompanied by increased upregulation of several costimulatory markers, which adds to its superior antitumor effect compared with that of NU-α-GalCer and α-GalCer. Crystallographic analysis revealed a previously unknown flexibility of the NKTCR footprint and therefore opens new avenues for the synthesis of novel Th1-polarizing glycolipids with therapeutic potential in cancer research.

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Disclosures

The authors have no financial conflicts of interest.

References