Synthesis and Immunological Activity of Novel Oligo(ethylene glycol) Analogues of α-Galactosylceramide

Mithun S. Pawar, Sravanthi Vemireddy, Shainy Sambyal, and Halmuthur M. Sampath Kumar*

ABSTRACT: CD1d-arbitrated activation of i-NKT cells by α-galactosylceramide results in the effective secretion of Th1 and Th2 cytokines, with adjuvanticity skewed toward Th2 immunity. However, the polarization of immune response could be achieved by suitable modification of the glycolipid structure. In the current study, novel glycolipids with an amphiphilic oligo ethylene glycol lipid moiety bearing the benzyloxy group at the terminus on the acyl arm of sphingosine, exhibited CD1d ligand binding as quantified by IL-2 cytokine production. When immunized with quadrivalent split influenza virus in BALB/c mice, the novel ceramide analogues with a longer oligo (ethylene glycol) chain length induced significant levels of antibody (IgG) with Th1-polarized immune response.

INTRODUCTION

α-Galactosylceramide (KRN7000; α-GalCer) is a synthetic glycolipid identified through structure–activity relationship (SAR) studies on a class of glycolipids first acquired from an marine sponge Agelas mauritianus from the Okinawan Sea of Japan.1−3 This ligand is known to induce a strong CD1d-dependent iNKT cell activation.4,5 Natural killer T (NKT) cells, being T-lymphocyte subsets, have properties of both T cells and NK cells. iNKT cells, a partial invariant surface receptor expressed by NKT cells, are capable of detecting the α-GalCer moiety with the aid of CD1d, a major histocompatibility complex (MHC) class-I-like protein. Upon stimulation of iNKT cells, IFN-γ (Th1) and IL-4 (Th2) cytokines are produced. GalCer’s anticanancer, antiviral/bacterial, and adjuvant actions are correlated to the production of Th1 cytokines, whereas Th2 cytokine secretion is linked with the improvement of conditions in some autoimmune illnesses.6−7 However, the efficiency of α-GalCer is often hampered due to inverse suppression of both Th1 and Th2 cytokines. Thus, the failure of GalCer in regressing firm tumors in a phase I trial may be attributed to the hampered therapeutic benefits of IFN-γ by IL-4, leaving no net advantage.8 Consequently, the development of compounds capable of inducing Th1/Th2 polarization to varying degrees is desirable.9,10 Attempts to specifically modulate the quick production of cytokines by NKT cells result in the production of numerous R-GalCer analogues, synthesized through modifications of the α-GalCer scaffold at suitable positions resulting in more potent derivatives, namely, C-GalCer, Nu-α-GalCer, OCH, RCAI-S6, 7DW8-S, EF77, SMC-124, and so forth;11−13 among various α-GalCer modifications that have been synthesized over the past several years, some compounds with potent immune stimulatory capability attracted our attention. An important variety among these analogues is α-GalCer derivatives that include a 1,2,3-triazole moiety replacing an amide linkage in the scaffold.14 The 1,2,3-triazolyl derivatives displayed an equivalent iNKT activation with a Th2 bias on mouse splenocytes. The triazole group being stable to hydrolytic cleavage assisted as a firm linker that is resistant to oxidative/reductive conditions in biological systems. α-GalCer analogues with various aryl moieties at the acyl chain terminus have elicited a remarkable activity, exhibiting strong iNKT activation with a strong Th1 bias. Docking studies revealed that supplementary hydrogen bonding between the phenyl at the terminus of the fatty acyl chain and the aromatic amino acid residues available in the A’ pocket in the CD1d hydrophobic groove would be more beneficial.15 One such derivative from the same structural group, 7DW8-S, elicited a superior adjuvant activity relative to that of α-GalCer when tested with malaria and HIV antigens in mice.16

In order to attain a better immune stimulation with a superior Th1 response, we have designed novel α-GalCer derivatives encompassing both the aforementioned structural elements. In this design, a benzyl group served as an aryl...
terminus of the alkyl chain, which in turn was attached to the azido-GalCer moiety with a triazole linker. The effectiveness of such a molecular design comprising benzyl and triazolyl moieties situated in the same structural scaffold in amending the immune response has been reported by us.\textsuperscript{17} To investigate the immunomodulatory propensities of these glycolipid moieties, we synthesized a focused library of α-GalCer derivatives wherein 1,2,3-triazole substituted the amide connection of glycolipids bearing lipid chains of varying lengths with some intervening oxygen atoms and a terminal benzyl group. These structural modifications are aimed at enhancing the hydrogen bonding through the iNKT cell activation, which may result in a stronger response skewed toward Th1. In persistence of our ongoing research program to develop novel immunomodulators, we showcase the synthesis and immunopharmacological studies of novel benzyloxyalkyl-substituted 1,2,3-triazolyl α-GalCer analogues.

\section*{RESULTS AND DISCUSSION}

\textbf{Ligand Design and Synthesis.} We synthesized a targeted library of 1,2,3-triazolyl α-GalCer derivatives bearing amphipathic oligoethylene lipid chains with a terminal benzyl group to investigate the immunomodulatory properties of these glycolipid moieties.

The synthesis of α-GalCer analogues was carried out using methodologies described in the literature, with a few minor changes. Two essential fragments (azido intermediate 8 and benzylated acids with varying chain lengths) were generated to achieve the synthesis of the desired analogues, and their syntheses are briefly explained below. Starting with β-D-galactose pentaacetate and following the previously published procedures, azido phytosphingosine 1 was synthesized\textsuperscript{18,19} (Scheme 1).

Azido phytosphingosine 1 was specifically protected as TBS silyl ether; then, the secondary hydroxyls were protected orthogonally with benzoyl (Bz) protection to yield compound 3, which may result in a stronger response skewed toward Th1. In persistence of our ongoing research program to develop novel immunomodulators, we showcase the synthesis and immunopharmacological studies of novel benzyloxyalkyl-substituted 1,2,3-triazolyl α-GalCer analogues.
chloride, yielding the benzoyl-protected azido intermediate 7 (Scheme 1).

The terminal benzylated acid intermediates 10a−e with varying chain lengths required for acid–amine coupling were prepared by monobenzylation of glycols 8a−e in DMF at room temperature (RT) with benzyl bromide (BnBr)/NaH, yielding monobenzylated alcohols 9a−e, which were then condensed with bromoacetic acid in the presence of sodium hydride to yield acid intermediates 10a−e, respectively. However, acid 11 was prepared by the condensation of benzyl alcohol with bromoacetic acid in the presence of NaH in DMF (Scheme 2).

The final constructs were made by the Staudinger reduction of azide 7 to amine 12 and then coupling amine with the previously synthesized acids (i.e., 10a−e and 11) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI)/hydroxybenzotriazole (HOBt) to yield 13−18, respectively. The final analogues 19−24 were obtained after deprotecting all benzoyl groups with NaOMe in MeOH. Each of these compounds was purified using column chromatography with CHCl3/methanol as eluents, with excellent yields (Scheme 3).

**Biological Evaluation.** All the new analogues were incubated with mouse splenocytes at varying concentrations of 10, 100, and 1000 ng/mL to determine their toxicity on the cells. After incubation, viability of the cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent, and from Figure 1 we can infer that all the analogues were non-cytotoxic even at a higher concentration of 1000 ng/mL and in turn have aided in proliferation of splenocytes. From the data, we can infer that the analogues were not cytotoxic at the concentrations considered for toxicity evaluation in further in vitro assays.

Initially, all the analogues were analyzed for their ability to bind to the Cd1D ligand using iNKT cells. iNKT cells were stimulated with all six analogues of GalCer and α-GalCer, incubated with protein Cd1D for 16 h, and analyzed for IL-2 production (Figure 2). The quantification of IL-2 cytokine in the in vitro study revealed that all six compounds were competent stimulators of iNKT cells. From the graph, we can interpret that the analogues with a shorter chain length such as 2EGBN-6OH and 3EGBN-6OH were active at a 1000 ng/mL concentration, but as the chain length increases, the analogues
4EGBN-6OH and SEG8N-6OH were equally active even at the lowest 10 ng/mL concentrations in comparison to standard α-GalCer. From the above data, we can interpret that as the chain length increases, the binding ability of the molecule is also enhanced, which resulted in enhanced binding which was evident with an increased production of IL-2 even at the lowest concentrations.

NKT cells of the immune system with a semi-invariant surface receptor (iNKT cells) exclusively identify galactoceramide presented by an MHC class-I-like molecule, which further leads to stimulation of the iNKT cells with an expression of IFN-γ, IL-2, and 4 cytokines. To determine the ability of these analogues to activate iNKT cells, splenocytes were incubated with three different concentrations of compounds, and cytokines were estimated from the supernatant after 48 h incubation. From Figure 3, we can observe a trend in all the three cytokines that analogues with a shorter chain length, such as PH-6A, EGBN-6OH, 2EGBN-6OH, and 3EGBN-6OH, have expressed higher cytokine production at a 1000 ng/mL concentration, but longer chain length analogues such as 4EGBN-6OH and SEG8N-6OH induced a significant cytokine production at a 100 ng/mL concentration when compared to that of standard GalCer. These data of the in vitro

Figure 2. In vitro Cd1D-iNKT binding assay.

Figure 3. In vitro cytokine estimation of IFN-γ, IL-2, and IL-4 from the splenocyte supernatant.
assay for iNKT cell activation are in line with those of the Cd1D binding assay, wherein we observe that analogues with a higher chain length have elicited a stronger Cd1D binding and iNKT activation.

After establishing the nontoxic nature and ligand binding and iNKT activation abilities of the ceramide analogues through primary in vitro assays, we proceeded further to evaluate the efficacy of these analogues in the in vivo model using BALB/c mice against quadrivalent flu antigen. MF59 and α-galactoceramide were considered as positive controls. Mice were immunized on day 0 and day 14 and sacrificed on day 28 to determine the adjuvant activity of analogues.

Antibodies (IgG, IgG1, and 2a) are considered as a hallmark of immune response. We have quantified the antibodies IgG and isotypes using sera from immunized mice groups using the indirect enzyme-linked immunosorbent assay (ELISA) method. From the data in Figure 4A we observe that all the analogues had significant antibody production compared to that in the antigen-alone group. However, when compared with the positive adjuvant control α-galactoceramide, the analogues 4 EGBN-6OH (5 μg, 1.5-fold) and 5 EGBN-6OH (20 μg, 1-fold) exhibited significant antibody production, with 10- and 6-folds in comparison to that in MF 59 and 10- and 7-folds in comparison to that in the antigen-alone group. These data give us an indication regarding the relation between the side chain length and enhancement of antibody production of the analogues.

Isotype quantification was carried out to determine the type of immune response Th1 or Th2, elicited by the analogues. From Figure 4B, we can see that the isotype titers of analogues are polarized toward Th1, whereas the parent compound α-GalCer (KRN7000) exhibited a profound Th2 response. It was also observed from the graph that as the concentration of the compound increases, that is, at 20 μg, a shift to Th2 response was observed with an increase in IgG1 antibody titers, whereas at lower concentrations, that is, 5 and 10 μg, a clear Th1-polarized immune response was noticed. A Th1-polarized

Figure 4. Antibody titers of IgG (A) of individual mice, where the bars are the mean ± standard deviation (SD) of titers and * = p ≤ 0.05 and ** = p ≤ 0.005 when compared to the antigen-alone group, and IgG 2a/G1 (B) of the immunized groups.
immune response is very much required for protection against viral and intracellular pathogens.

Cytokine quantification was carried out using the sandwich ELISA method wherein stimulated cell supernatants were used for IFN-γ, IL-2, and IL-4 cytokine estimation. From Figure 5 we can observe a trend wherein both 4EGBN-6OH and 5EGBN-6OH analogues have stimulated significant IFN-γ and IL-4 cytokine responses at a lower concentration (5 and 10 μg). These results for Th1 cytokine production (IFN-γ and IL-2) indicate that the antibacterial and antiviral adjuvant activities of the analogues, when compared to those of the standard α-GalCer, were significant.

**Immunophenotyping.** Splenocytes from immunized mice were used for determination of CD4, 8, and 19 markers. From Figure 6 we can observe that both CD4 and CD8 populations enhanced significantly compared to those in the antigen-alone group but were comparable to those of GalCer. However, the B cell population of the immunized groups enhanced but not significantly compared to that of the antigen group.
To sum up, the novel oligo (ethylene glycol) analogues of α-GalCer exhibit potential immune modulatory properties arising from their capability to bind to Cd1D-iNKT and activate the Th1-mediated immune response as evidenced from isotype and antibody titers along with high levels of IFN-γ, IL-2, and 4 cytokine expressions. Increased expressions of CD4 and CD8 T cell markers indicate enhanced T-helper cell and cytotoxic T-lymphocyte activation. Overall, the novel GalCer analogues

Figure 6. (A) Immunophenotyping of immunized mice splenocytes for quantification of CD4 and 8 populations. (B) Bar graph representing % population of B cells quantified using flow cytometry.

CONCLUSIONS
induce a qualitatively superior immune response with a Th1 bias, which is much required to protect from the intracellular pathogenesis-like viral infections such as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) wherein a potent T-cell-mediated response together with the humoral response is essential.

**METHODS**

**iNKT Isolation.** The invariant natural killer T-cell isolation kit (MACS, Miltenyi Biotec, Germany) is a quick enrichment kit for aseptic isolation of all NKT cell subpopulations. Isolation of cells was performed as per the manufacturer’s instructions with minor modifications. Briefly, cells were made up 1 × 10^5 cells/mL in the range of 0.5–2.0 mL, and the sample was added to 5.0 mL tubes. A mouse iNKT cell biotin-antibody cocktail (50 μL/mL) was added to the cells and then mixed well and incubated at 2–8 °C for 15 min. After incubation, the cells were washed with the recommended medium and centrifuged at 300g for 10 min. The supernatant was discarded, and the pellet was resuspended in the original volume of 0.5–2.0 mL in a recommended medium. The magnetic particles were vortexed, and 75 μL/mL of the particles was added to the cells, which was then mixed and incubated at 2–8 °C for 10 min. The recommended medium was added to the sample and mixed gently by pipetting up and down. The tubes, without lids, were inserted into the magnet and incubated at RT for 5 min. The magnet was lifted and at once inverted with tube, pouring the entire enriched cell suspension into a new tube. The above-mentioned step was repeated one more time, and the isolated cells were ready for use.

**CD1d-iNKT In Vitro Assay.** Briefly, CD1d protein was coated in a 96-well plate at a concentration of 1 μg/100 μL in phosphate-buffered saline (PBS) and kept for incubation for 1 h at 37 °C. The plates were then washed with PBS and blocked by incubation for 1 h at 37 °C with 10% fetal calf serum (FCS) in PBS. After washing, the compounds were added at a concentration of 10, 100, and 1000 ng/mL and further incubated for 16 h at 37 °C. The plates were then washed using the complete RPMI medium, and fresh isolated NKT cells (3 × 10^5 cells per well) were added. After 16 h, the release of IL-2 was measured by sandwich ELISA.

**In Vitro Stimulation Assay.** The splenocytes were isolated and cultured in RPMI 1640 media and seeded at a density of 1 × 10^5 cells/well in 96-well plates. The cells were treated with varying concentrations of GalCer analogues (1000, 100, and 10 ng/mL) and incubated for 48 h at 37 °C; after incubation, the supernatant was collected and cytokine-like IFN-γ, IL-4, and IL-2 were quantified using the sandwich ELISA method.

**Splenocyte Proliferation Assay.** The splenocytes were isolated and cultured in RPMI 1640 media and seeded at a density of 1 × 10^5 cells/well in 96-well plates. The cells were treated with varying concentrations of GalCer analogues (1000, 100, and 10 ng/mL) and incubated for 48 h at 37 °C; after incubation, 20 μL of the MTT reagent (5 mg/mL) per well was added, and after 2 h incubation, 100 μL of media was discarded and compensated by 100 μL of DMSO (Sigma-Aldrich, France), followed by incubation at RT for 15 min. The absorbance was measured using a multimode reader (TECAN-Infinite M200 Pro) at 630 nm. Cytotoxicity of the compounds was determined by considering cell controls as 100% viable.

% Viability = OD of treated cells/ OD of untreated cells × 100

**In Vivo Immunization.** In vivo evaluation of the analogues was carried out in BALB/c mice (n = 5) with quadrivalent split influenza virus antigen. All animal experimentation was carried out according to institutional ethics and guidelines (IAEC no. IICT/IAEC/056/2021). Briefly, mice were divided into groups and administered intramuscularly with the quadrivalent flu antigen (Vaxiflu-4) alone (1 μg/mice); MF 59 adjuvant with antigen (1 μg/mice); and GalCer analogues at 2, 5, and 10 μg/mice along with standard galactoceramide with a 10 μg/mice dose on the 0th day, and a booster was given on the 14th day. After the 28th day, blood from immunized mice was collected for antibody titrations, and spleens were harvested by sacrificing the mice to measure other immunological parameters as discussed below.

**Antibody Titters.** The serum from the immunized mouse was collected, and IgG, IgG1, and IgG2a antibodies were quantified by the indirect ELISA method. Briefly, plates were coated with antigen in carbonate buffer and incubated at 4 °C overnight and then washed with PBS with Tween (PBST) and blocked with 1% bovine serum albumin (BSA) solution for 1 h and then washed thrice. Twofold diluted serum samples were added and incubated for 3 h at 37 °C. After incubation, plates were added with HRP-conjugated IgG, IgG1, and IgG2a antibodies at 1:3000 dilution and incubated for 30 min at RT. After washing the plates, a 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and incubated for 15–30 min in the dark, and the reaction was stopped using 1 N H2SO4. Thereafter, the plates were read at 450 nm using the multimode reader, and titers were calculated using the absorbance recorded.

**Cytokine Estimation.** IFN-γ, IL-4, and IL-2 cytokines were estimated using sandwich ELISA from the supernatant collected from antigen restimulated splenocytes of immunized mice. Briefly, plates were coated with the capture antibody in carbonate buffer, incubated overnight at 4 °C, and then blocked with 1% BSA and incubated for 1 h at RT. Plates were washed, and a cell supernatant was added and incubated for 2 h, and then, a purified antibody was added. Plates were washed followed by the addition of the TMB substrate for quantification and read at 450 nm after stopping the reaction with 1 N H2SO4 using the multimode reader.

**Immunophenotyping.** A single cell suspension of the spleen from immunized mice was prepared, and 3 × 10^5 cells/group were taken in a tube and washed with PBS. Then, 1 μL/tube of the Fc blocker was added and incubated for 15 min, followed by washing. Cells were added with fluorochrome-conjugated anti-CD4, -CD8, and -CD 19 antibodies and incubated for 45 min in the dark. Cells were washed twice and resuspended in sheath buffer for flow cytometric analysis. Cells were acquired using FACSVerse and analyzed using BD FACSuite software.

**MTT Assay.** Spleen cells from immunized mice were seeded at a density of 1 × 10^5 cells/well in a 96-well plate using RPMI 1640 media. Then, cells were treated with antigen alone (0.1 μg/mL) and mitogens-like lipopolysaccharide (LPS) (10 μg/mL) and concanavalin A (ConA) (2 μg/mL) and incubated for 48 h at 37 °C. After incubation, 20 μL of the MTT reagent was added to the wells and incubated further for 3 h. After incubation, cells were centrifuged, and the supernatant was removed; DMSO was added to crystallize MTT and incubated
for 15 min in the dark. The plate was read at 630 nm using the multimode reader, and splenocyte proliferation was calculated from the absorbance.\(^\text{24}\)

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02125.

Synthetic experimental procedures, compounds’ data, and \(^1\)H and \(^{13}\)C NMR spectra of compounds (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Halmuthur M. Sampath Kumar — Vaccine Immunology Laboratory, OSPC Division, CSIR-Indian Institute of Chemical Technology, Hyderabad 500007, India; Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh 201 002, India; orcid.org/0000-0002-4473-4220; Phone: +914027191824; Email: sampath.iict@gov.in; Fax: +91-40-27160387

**Authors**

Mithun S. Pawar — Vaccine Immunology Laboratory, OSPC Division, CSIR-Indian Institute of Chemical Technology, Hyderabad 500007, India; Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh 201 002, India

Sravanthi Venireddy — Vaccine Immunology Laboratory, OSPC Division, CSIR-Indian Institute of Chemical Technology, Hyderabad 500007, India

Shainy Sambyal — Vaccine Immunology Laboratory, OSPC Division, CSIR-Indian Institute of Chemical Technology, Hyderabad 500007, India; Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh 201 002, India

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsomega.2c02125

**Author Contributions**

M.S.P. equal author contribution. M.S.P. performed synthesis, purification, and spectral characterization of GalCer analogues. S.V. performed preclinical immunopharmacological evaluation, data collection, data analysis, and drafted the manuscript. S.S. performed immunopharmacological evaluation. H.M.S.K. conceived the idea and performed editing and critical revisions of the manuscript.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Authors thank DBT for the financial support under the INDIGO program, and M.P. and S.S. thank UGC, India, and DST for the award of fellowships. IICT manuscript communication no. IICT/Pubs./2022/029.

**REFERENCES**

(1) Morita, M.; Motoki, K.; Akimoto, K.; Natori, T.; Sakai, T.; Sawa, E.; Yamaji, K.; Koezuka, Y.; Kobayashi, E.; Fukushima, H. Structure-Activity Relationship of \(\alpha\)-Galactosylceramides against B16-Bearing Mice. *J. Med. Chem.* 1995, 38, 2176–2187.

(2) Natori, T.; Koezuka, Y.; Higa, T. Agelasphins, novel \(\alpha\)-galactosylceramides from the marine sponge Agelas mauritianus. *Tetrahedron Lett.* 1993, 34, 5591–5592.

(3) Togashi, Y.; Chamoto, K.; Wakita, D.; Tsutsumi, N.; Iwakura, Y.; Matsubara, N.; Kitamura, H.; Nishimura, T. Natural killer T cells from interleukin-4-deficient mice are defective in early interferon-\(\gamma\) production in response to \(\alpha\)-galactosylceramide. *Cancer Sci.* 2007, 98, 721–725.

(4) Fujii, S.-i.; Shimizu, K.; Smith, C.; Bonifaz, L.; Steinman, R. M. Activation of natural killer T cells by \(\alpha\)-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J. Exp. Med.* 2003, 198, 267–279.

(5) Sada-Ovalle, I.; Sköld, M.; Tian, T.; Besra, G. S.; Behar, S. M.; medicine, c. c. α-Galactosylceramide as a therapeutic agent for pulmonary Mycobacterium tuberculosis infection. *Am. J. Respir. Crit. Care Med.* 2010, 182, 841–847.

(6) Giaccone, G.; Punt, C. J.; Ando, Y.; Ruitier, R.; Nishi, N.; Peters, M.; Von Blomberg, B. M. E.; Scheper, R. J.; Van der Vliet, H. J.; Van den Eertwegh, A. J. M. A phase I study of the natural killer T-cell ligand \(\alpha\)-galactosylceramide (KRN7000) in patients with solid tumors. *Clin. Cancer Res.* 2002, 8, 3702–3709.

(7) Matsuda, J. L.; Mallevaej, T.; Scott-Browne, J.; Gapin, L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr. Opin. Immunol.* 2008, 20, 358–368.

(8) Godfrey, D. I.; MacDonald, H. R.; Kronenberg, M.; Smyth, M. J.; Kaer, L. V. \(\alpha\)NKT cells: what’s in a name? *Nat. Rev. Immunol.* 2004, 4, 231–237.

(9) Smyth, M. J.; Godfrey, D. I. NKT cells and tumor immunity—a double-edged sword. *Nat. Immunol.* 2000, 1, 459–460.

(10) Berkers, C. R.; Ovaa, H. Immunotherapeutic potential for ceramide-based activators of iNKT cells. *Trends Pharmacol. Sci.* 2005, 26, 252–257.

(11) Miyamoto, K.; Miyake, S.; Yamamura, T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing Th 2 bias of natural killer T cells. *Nature 2001*, 413, 531–534.

(12) Franck, R. W. C-galactosylceramide: Synthesis and immunology. *Acc. Chem. Res.* 2012, 45, 46–56.

(13) Aspeslagh, S.; Li, Y.; Yu, E. D.; Pauwels, N.; Trappeners, M.; Girardi, E.; Decruy, T.; Van Beneden, K.; Venken, K.; Drennan, M.; Leybaert, L.; Wang, J.; Franck, R. W.; Van Calenbergh, S.; Zajonc, D. M.; Elewaut, D. Galactose-modified iNKT cell agonists stabilized by an induced fit of CD1d prevent tumour metastasis. *EMBO J.* 2011, 30, 2294–2305.

(14) Lee, T.; Cho, M.; Ko, S.-Y.; Youn, H.-J.; Baek, D. J.; Cho, W.-J.; Kang, C.-Y.; Kim, S. Synthesis and evaluation of 1,2,3-triazole containing analogues of the immunostimulant alpha-GaCer. *J. Med. Chem.* 2007, 50, 585–589.

(15) Wu, D.; Zajonc, D. M.; Fujio, M.; Sullivan, B. A.; Kinjo, Y.; Kronenberg, M.; Wilson, I. A.; Wong, C.-H. Design of natural killer T cell activators: structure and function of a microbial glycosphingolipid bound to mouse CD1d. *Proc. Natl. Acad. Sci. U.S.A.* 2006, 103, 3972–3977.

(16) Padte, N. N.; Boente-Carrera, M.; Andrews, C. D.; McManus, J.; Graspe, B. F.; Gettie, A.; Coelho-dos-Reis, J. G.; Li, X.; Wu, D.; Bruder, J. T.; Sedegah, M.; Patterson, N.; Richie, T. L.; Wong, C.-H.; Ho, D. D.; Vasan, S.; Tsuji, M. A glycolipid adjuvant, 7DW8-5, enhances CD8\(^+\) T cell responses induced by an adenovirus-vectored malaria vaccine in non-human primates. *PLoS One* 2013, 8, No. e78407.

(17) Verma, Y. K.; Reddy, B. S.; Pawar, M. S.; Bhunia, D.; Sampath Kumar, H. M. Design, Synthesis, and Immunological Evaluation of Benzyloxyalkyl-Substituted 1,2,3-Triazolyl \(\alpha\)-GaCer Analogues. *ACS Med. Chem. Lett.* 2016, 7, 172–176.

(18) Asai, N.; Fusetani, N.; Matsunaga, S. Sex Pheromones of the Hair Crab Erimacrus isenbeckii. II. Synthesis of Ceramides. *J. Nat. Prod.* 2001, 64, 1210–1215.

(19) León, F.; Brouard, I.; Rivera, A.; Torres, F.; Rubio, S.; Quintana, J.; Estévez, F.; Bermejo, J. Isolation, structure elucidation,
total synthesis, and evaluation of new natural and synthetic ceramides on human SK-MEL-1 melanoma cells. *J. Med. Chem.* 2006, 49, 5830−5839.

(20) Sant, S.; Poulin, S.; Hildgen, P. Effect of polymer architecture on surface properties, plasma protein adsorption, and cellular interactions of pegylated nanoparticles. *J. Biomed. Mater. Res., Part A* 2008, 87A, 885−895.

(21) van der Heyde, H. C.; Burns, J. M.; Weidanz, W. P.; Horn, J.; Gramaglia, I.; Nolan, J. P. Analysis of antigen-specific antibodies and their isotypes in experimental malaria. *Cytometry, Part A* 2007, 71A, 242−250.

(22) Ledur, A.; Fitting, C.; David, B.; Hamberger, C.; Cavaillon, J.-M. Variable estimates of cytokine levels produced by commercial ELISA kits: results using international cytokine standards. *J. Immunol. Methods* 1995, 186, 171−179.

(23) Gandhapudi, S. K.; Ward, M.; Bush, J. P. C.; Bedu-Addo, F.; Conn, G.; Woodward, J. G. Antigen priming with enantiospecific cationic lipid nanoparticles induces potent antitumor CTL responses through novel induction of a type I IFN response. *J. Immunol.* 2019, 202, 3524−3536.

(24) Preethi Pallavi, M. C.; Bhunia, D.; Srinivasa Reddy, B.; Sravanthi, V.; Sampath Kumar, H. Novel Mannosylated Synthetic Saponin as Vaccine Adjuvant for Recombinant Japanese Encephalitis Antigen. *J. Immunol. Forecast.* 2018, 1, 1002.