Potent Neutralizing Antibodies Elicited by RBD-Fc-Based COVID-19 Vaccine Candidate Adjuvanted by the Th2-Skewing iNKT Cell Agonist

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ABSTRACT: The development of a safe and effective COVID-19 vaccine is of paramount importance to terminate the current pandemic. An adjuvant is crucial for improving the efficacy of the subunit COVID19 vaccine. α-Galactosylceramide (αGC) is a classical iNKT cell agonist which causes the rapid production of Th1- and Th2-associated cytokines; we, therefore, expect that the Th1- or Th2-skewing analogues of αGC can better enhance the immunogenicity of the receptor-binding domain in the spike protein of SARS-CoV-2 fused with the Fc region of human IgG (RBD-Fc). Herein, we developed a universal synthetic route to the Th1-biasing (α-C-GC) and Th2-biasing (OCH and C20:2) analogues. Immunization of mice demonstrated that αGC-adjuvanted RBD-Fc elicited a more potent humoral response than that observed with Alum and enabled the sparing of antigens. Remarkably, at a low dose of the RBD-Fc protein (2 μg), the Th2-biasing agonist C20:2 induced a significantly higher titer of the neutralizing antibody than that of Alum.

INTRODUCTION

The pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, has led to global social and economic disruption as well as substantial healthcare challenges. The development of a safe and effective vaccine is of paramount importance to terminate this pandemic, particularly as variants of SARS-CoV-2 continue to evolve.1 To date, various vaccine types have been developed. Compared to RNA vaccines and adenovirus vector vaccines that we have limited experience with, the protein-based subunit vaccines are well understood with a proven track record of safety and efficacy in many infectious diseases.6

The receptor-binding domain (RBD) in the spike (S) protein of SARS-CoV-2 mediates viral entry during the initial infection through its binding to the angiotensin-converting enzyme 2 (ACE2) receptor. The computational study showed that RBD, unlike other parts of S proteins, is not extensively shielded by glycans from antibody recognition.3 The glycosylation sites were located in the RBD core subdomain and were found to be distant from the area bound to ACE2.4 Wang and co-workers, through the chemical synthesis of homogeneous glycoforms of RBD, showed that the glycosylation on the RBDs does not impact their binding with ACE2.5 Therefore, RBD serves as an important antigen for the development of COVID-19 subunit vaccines, and numerous researchers revealed the great potential of RBD-based subunit vaccines.6−10 However, the use of RBD in vaccines is impaired by its poor immunogenicity owing to its small molecular size. In addition, RBD tends to establish a monomer−dimer equilibrium in solution by forming the disulfide bond at the cysteine residue C603, and there is a sharp difference in the immunogenicity of the RBD monomer and dimer.7 One solution to address these limitations is to fuse the C-terminus of RBD with the Fc fragment of human IgG, generating RBD-Fc with improved in vivo stability and immunoglobulin-mediated effector function.11−13 Importantly, the RBD-Fc-based vaccine can provide protection against SARS-CoV-2 with several natural mutations in RBD,4,14,15 including the N501Y and D614G mutations. These findings are valuable concerning the emergence of SARS-CoV-2 variants globally. A recombinant vaccine containing an RBD-Fc fusion (RBD-Fc Vacc) is currently being assessed in phase I/II human clinical trials.4

Apart from the identification of suitable antigens, an equally crucial aspect of subunit vaccines is the selection of appropriate

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adjuvants, which can augment the magnitude, quality, and durability of the immune responses induced by vaccination even with lower doses of the antigen. The most widely used adjuvant, an aluminum salt (Alum), has been the only human vaccine adjuvant used over the last century. Substantial effort has been expended to choose a highly effective adjuvant for the RBD-based subunit COVID-19 vaccine.18,19 α-galactosylceramide (αGC) is an invariant natural killer T (iNKT)-cell ligand whose adjuvant activity has aroused great research interest over the past 2 decades.20 iNKT cells are a subset of T lymphocytes which are specifically activated by glycolipid antigens presented by the atypical MHC class I molecule CD1d. Upon activation, iNKT cells rapidly secrete abundant amounts of T helper (Th) 1, Th2, and Th17 cytokines and tailor the immune response through the subsequent activation of dendritic, NK, T, and B cells. As a classical iNKT glycolipid agonist, αGC demonstrates excellent immunological activity in animal models, but the elicitation of the concomitant secretion of Th1 and Th2 cytokines, which have opposing functions in vivo, is believed to limit the clinical outcome of αGC-based immunotherapy. Consequently, some analogues of αGC were developed that can alter the downstream cytokine response, skewing it toward either a more pronounced Th1 or a Th2 phenotype. The most extensively studied Th1-biasing agonist is α-C-GC (Scheme 1B), in which the oxygen of the glycosidic bond was replaced with a methylene group.21 α-C-GC demonstrated a highly improved potency over αGC, a 1000-fold more potent antimalarial activity and a 100-fold more potent antimetastatic activity in the mouse models of malaria and melanoma metastases, respectively.22 OCH, an analogue of αGC with a truncated sphingosine side chain, induces a predominant Th2 response in vivo.23 C20:2 contains a C20-acyl chain with a diene and has been identified as an exceptionally potent iNKT cell activator that delivered a markedly Th2-biased cytokine response in mice and had potent anti-inflammatory properties.24

The adjuvant activity of αGC and analogues have been explored in the vaccines against influenza25 and HIV.26–31 Notably, αGC was shown to have a dose-sparing effect32 and, equally important, αGC is also capable of promoting a rapid rise in serum IgG after one immunization.33 These two properties may be valuable during a pandemic of an emerging infectious disease, such as COVID-19. Therefore, in the present study, we began the immunological study by examining the adjuvant activity of αGC in subunit vaccine candidates with different doses of the RBD-Fc protein (Scheme 1C). The efficacy of vaccine candidates was identified by SARS-CoV-2 RBD-specific humoral immune responses, particularly neutralizing antibodies that are the major immune correlates of protection. Because of an unbiased Th0-associated cytokine profile for αGC, we also determined whether the Th1- or Th2-skewing analogues of αGC can stimulate a more effective humoral immune response than αGC. Furthermore, given the fact that the delivery system of αGC plays an important role in optimizing NKT cell-based immune responses,34 we investigated the impact of the liposomal formulation of αGC-based glycolipids on their adjuvant efficacy. On the other hand, to access the iNKT agonists needed here, we developed a universal synthetic route to αGC and the representative analogues (α-C-GC, OCH, and C20:2). The key steps involving olefin cross-metathesis, Sharpless asymmetric epoxidation, and ring opening of epoxides with an azide enable the construction of the three consecutive stereocenters in the sphingosine chain. The stereochemistry of epoxide opening was checked.

**RESULTS AND DISCUSSION**

**Synthesis of α-C-GC.** Since the first synthesis of α-C-GC (2),35 a variety of efficient synthetic routes have been developed due to its remarkable immunological activity.36–43 Our current synthetic route is similar to that of α-1C-GalCer that induces Th1-biased responses in human NKT cells.44,45 As illustrated in Scheme 2, our synthesis of 2 commenced with one-carbon homologation of 5 via hydroboration, Dess–Martin oxidation, and Wittig olefination to form C-allyl glycosides 6 with 74% yield over three steps. Olefin cross-metathesis of 6 with the optically active allyl alcohol 7 proceeds to afford allylic alcohol 8 after deacetylation with NaOMe. Sharpless asymmetric epoxidation37 of 8 provides epoxy alcohol 9 with high diastereoselectivity (dr > 20:1). Chelation-controlled opening of 9 with NaN₃/NH₄Cl in aqueous MeOH under reflux delivered the desired azido diol 10 in a high yield (98%). One unidentified side product (yield < 5%) was isolated from the mixture. Notably, 10 exhibited the same Rₙ value to reactant 9 on the thin-layer chromatography (TLC) plate with various eluents, and the reaction progress had to be monitored with the ¹H NMR. The protection of diol 10 under the condition of 2,2-DMP/PPTS provides

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**Scheme 1.** RBD-Fc, iNKT Cell Agonists, and Immunization Protocol

(A) RBD-Fc: a recombinant fusion protein generated by fusing the C-terminus of SARS-CoV-2 spike RBD to a human IgG1 Fc region. (B) Glycolipid Agonists of iNKT Cells: αGC (1), α-C-GC (2), OCH (3), and C20:2 (4). (C) Mouse immunization protocol.
isopropylidene 11. Subsequently, the Staudinger reduction of the azido group with PMe$_3$ and N-acylation with n-hexacosanoyl chloride afforded amide 12, which underwent acid hydrolysis and hydrogenolysis to deliver α-C-GC (2).

**Synthesis of αGC, OCH, and C20:2.** Many synthetic routes to α-GC take the advantage of using commercially available phytosphingosine. Recently, a large-scale synthesis of α-GC has been developed. However, the synthetic routes using phytosphingosine as a starting material cannot be used to prepare OCH (3). Given the successful preparation of α-C-GC (2), we carried out the synthesis of O-glycosides via the same synthetic sequence. As illustrated in Scheme 3, our first goal is to prepare 14 as the reactant of olefin metathesis. Owing to the toxicity of allyl alcohol, 3-bromo-1-propanol was instead employed as the glycosyl acceptor. The DMF-mediated glycosylation of 13 with 3-bromo-1-propanol afforded the α-glycoconjugate, which then provided the allyl galactoside 14 after elimination with t-BuOK. Similar to the synthesis of α-C-GC, the three-step transformation of 14 into 16-αGC or 16-OCH was uneventful. However, the ring opening of epoxide 16-OCH affords the azidodiol 17-OCH, together with an almost equal amount of regioisomers 18-OCH that was separated with column chromatography. Analogously, the conversion of 16-αGC to 17-αGC also gave rise to poor regioselectivity. The vanished regioselectivity might be have a similar electron-withdrawing inductive effect on both sides of the epoxide. Benzylidene of 17-αGC or 17-OCH gave rise to 19-αGC or 19-OCH, respectively. After Staudinger reduction of 19-αGC or 19-OCH, the completion of the synthesis achieved through N-acylation with n-hexacosanoyl chloride and global deprotection to deliver α-GC (1) or OCH (3).

The synthetic route to C20:2 started from 21 that was prepared by the acidic hydrolysis of benzylidene in 20-αGC. Attempt to transform 21 into 22 within one step under the condition of H$_2$/Pearlmann’s catalyst was unsuccessful. Therefore, a two-step conversion, Staudinger reduction of 21 followed hydrogenolysis of all benzyl protecting groups, was adopted to afford 22, which was N-acylated by 8,11-eicosadienoic chloride to provide C20:2 (4) in 65% yield over three steps.

**Confirmation of the Stereochemistry of Epoxide Opening.** We confirmed the configuration at C-3 by the advanced Mosher method. As shown in Scheme 4A, Mosher amides were prepared by the reaction of (S)-(+) α-methoxy-α-(trifluoromethyl)phenylefacyl chloride (MTPA) chloride or (R)-MTPA chloride with amine, which was prepared by the reduction of the azido group in 10. Due to the overlap of crucial signals in the $^1$H NMR spectrum, further derivatization was conducted to provide 24S and 24R. $^1$H NMR analysis of the corresponding (S)- and (R)-Mosher amides reveals the difference between the chemical shifts of the H$_3$ in the (R)- and (S)-MTPA ester. The upfield signal of H$_3$ in the (S)-MTPA ester 24S ($\delta$$_R$ = 4.99 ppm) compared to that in the (R)-MTPA ester 24R ($\delta$$_R$ = 4.89 ppm) indicates that 10 has the R configuration ($\delta$$_L$ – $\delta$$_R$ = +0.10 ppm) at the C-3 position. In addition, the $^1$H and $^{13}$C NMR spectra of α-C-GC prepared by our new route are identical to those of the authentic sample but are different from the C-3 epimer of α-C-GC, further excluding the intramolecular participation of oxygen in the step of epoxide opening.

Fraser-Reid and Mootoo reported that oxygens present in ethers and pyranose rings can participate in electrophilic reactions at remote centers via five- and six-membered heterocycles. In fact, Franck and Pu did observe the retention of configuration at the azide-bearing carbon during the step of epoxide opening by azide as a nucleophile. Two mechanisms could cause the double inversion of the C-3 position (Scheme 4B): intramolecular participation by galactosyl ring oxygen (route i) or the 2′-O-benzyl group (route ii) to form an oxonium ion intermediate, which on an attack by the azide would form azido diol 10′ with the retention of stereoconfiguration.

Unlike the high regioselectivity in the step of epoxide opening in the synthesis of C-glycoside, the epoxide opening in the synthesis of O-glycoside gave rise to a mixture of regioisomers. We confirmed the regiochemistry by $^{13}$C NMR analysis of acetonides 25 and 26, which were prepared by the treatment of 17-OCH and 18-OCH with 2,2-dimethoxypropane (DMP), respectively. The quaternary ketal carbon signal ($\delta$ 108 and $\delta$ 101 ppm) is a characteristic of a five- and six-membered acetonide, respectively. Unambiguously indicated the relationship of the two hydroxy groups (Scheme 4C).

**αGC-Adjuvanted RBD-Fc Protein Elicited a More Potent Anti-RBD Antibody Response than Alum.** To evaluate the antibody responses elicited by the αGC-adjuvanted RBD-Fc protein, we immunized BALB/c mice three times at 2 week intervals, and sera were collected on day 0 and 14 days after each vaccination, according to the protocol:

**Scheme 2. Synthesis of C-Glycoside α-C-GC (2)**

- a) S-BBN; then NaOH, H$_2$O$_2$
- b) Dess-Martin periodinane
- c) Ph$_3$PCH$_2$Br, t-BuOK
- d) Grubbs cat. 2nd generation CH$_2$Cl$_2$, reflux, 2 h
- e) NaOMe, MeOH/CH$_2$Cl$_2$ (1:1)
- f) D-(-) DiPT Ti(O-iPr)$_4$, PhMe$_2$CO/CH$_2$Cl$_2$
- g) Na$_2$S$_2$, NH$_4$Cl, MeOH/H$_2$O (8:1)
- h) 2,2-DMP PPTS
- i) PMe$_3$, MeOH
- j) C$_2$H$_5$O$_3$, Et$_3$N, CH$_2$Cl$_2$/MeOH (2:1)
- k) TFA/H$_2$O (4:1), 10 min
- l) H$_2$, Pd/C, CH$_2$Cl$_2$/MeOH (2:1)
Alum was chosen as a positive control because it is a time-honored adjuvant that has been considered as the “gold standard” against which new adjuvant candidates are compared.17 The negative control animals were administered with RBD-Fc alone. Given the global scale need for immunization against SARS-CoV-2, we sought to determine the potential for αGC to enable antigen dose sparing, three different dose levels of the RBD-Fc protein (2, 8, and 32 μg, respectively) were admixed with adjuvants (Table S2). The subcutaneous injections, rather than intraperitoneal or intravenous injections, were adopted to avoid or reduce the impact of iNKT cell anergy.59 2 μg of αGC was administrated because this amount will not decrease the frequency of splenic or lymph node iNKT cells.59 Although a more potent immune response was found to be induced by cationic liposomes,60 we chose to formulate αGC in non-cationic liposomes to avoid cytotoxicity and uncontrolled immune responses, especially regarding the high safety demand on COVID-19 vaccines.

A strong correlation has been found between spike-pseudo-typed neutralization assay and protection from a SARS-CoV-2 challenge in non-human primates.19 Neutralizing activity was assessed through the measurement of pseudovirus neutralization titers at the half-maximal inhibitory concentration (IC50). As shown in Figure 1A and Table S7, analysis of the neutralizing antibody level on day 42 reveals that the αGC-adjuvanted group at the 32 and 8 μg of antigen dose levels elicit significantly enhanced neutralizing titers, at least 5-fold greater than those observed in cohorts that receive dose-matched RBD-Fc alone. Furthermore, the level of the neutralizing antibody in mice vaccinated with 8 μg of RBD-Fc in the presence of αGC was 2.3-fold higher than that in mice vaccinated with 32 μg of RBD-Fc in the absence of any adjuvant, although this difference did not reach statistical significance, indicating a significant potential for αGC to enable dose sparing of the RBD-Fc protein. In contrast, Alum induced only mildly increased titers of the neutralizing antibody compared to the immunization of RBD-Fc with no adjuvant, and no statistical difference was observed between Alum and no adjuvant for all three doses of RBD-Fc. It is worth mentioning that when 32 μg of RBD-Fc was administrated, the neutralizing antibody titer was significantly higher for αGC-treated mice compared with Alum tested, suggestive of the greater adjuvant activity of αGC than Alum.

A pseudovirus neutralization assay on sera collected on day 28 indicated that the vaccination of 2 μg of the RBD-Fc protein adjuvanted by αGC achieved approximate 1450 titer of the neutralizing antibody after second immunization, although there is no statistical significance compared to other two dose-matched comparators, this same level of neutralization was accomplished by Alum with 8 μg of the RBD-Fc protein after third immunization (on day 42) or Alum with 32 μg of the RBD-Fc protein on day 28 (Figure 1A and Table S6), indicating, to some extent, the rapid production of an effective antibody response as well as dose-sparing effect offered by αGC.
Remarkably, the increased dose of RBD-Fc alone or combined with Alum did not induce a significantly higher level of the neutralizing antibody, consistent with the report on MERS coronavirus RBD-Fc. However, αGC demonstrated a marked dose-dependent effect, probably attributable to more effective interactions between RBD-Fc and αGC.

Total IgG titers were measured using the protein of RBD-His, rather than RBD-Fc, to coat the wells of plates because the immunized mice could produce anti-human Fc antibodies. Total IgG titers were measured using the protein of RBD-His, rather than RBD-Fc, to coat the wells of plates because the immunized mice could produce anti-human Fc antibodies.14

IgG responses exhibited similar trends to the spike-pseudotyped neutralization assay (Figure 1B). Isotype analysis gave rise to those similar observations to those made against the TT antigen, with αGC not significantly polarizing the Th response compared with Alum (Figure S8). Additionally, αGC induced mainly the IgG1 antibody, a trace amount of IgG2a, IgG2b, and IgG3 antibodies, being in good agreement with previous reports by Wong and co-workers. However, the relatively lower level of IgG3 compared to IgG1 is in stark contrast with the similar hierarchy of IgG1 and IgG3 induced by the vaccines against carbohydrate antigens. This difference might be attributed mainly to the existence of the T helper epitope in the RBD-Fc region, which is contained in vaccine candidates.

RBD-Fc Protein Adjuvanted by Th2-Skewing Agonists Elicited Higher Titers of the Neutralizing Antibody. To determine whether the Th1- or Th2-skewing agonist is capable of promoting a more potent neutralizing antibody, especially in the presence of the low dose antigen, we immunized the mice with 2μg of the RBD-Fc protein coformulated as liposomes with 2μg of αGC or equal mols of α-C-GC, OCH, or C20:2 (Table S3). Alum was used as a control. The anti-sera on day 42 was used to determine the level of neutralizing antibodies. As illustrated in Figure 2A,B, very high pseudovirus neutralizing antibody responses are observed in mice immunized with 2μg of RBD-Fc adjuvanted with OCH; high responses are observed with C20:2 and αGC; and moderate responses are observed with α-C-GC and Alum. Notably, despite the magnitude being not the highest, the Th2 skewing agonist C20:2 demonstrates a significant degree to Alum. Our data suggested that the Th2-skewing agonists, combined with a low level of the antigen, represent an optimal category of the glycolipid in stimulating the generation of neutralizing antibodies, greatly outperforming Alum.

Total IgG titers (Figure 2C) are correlated to neutralizing antibodies, except for the OCH group, probably because OCH can induce a more effective antibody affinity maturation. It is noteworthy that αGC can rapidly elicit RBD-specific IgG up to a high level of endpoint titer (∼5.0 × 105) after second immunization, but the IgG level after third immunization was marginally increased (1.3-fold). In contrast, the third immunization of C20:2-treated mice can enable a 4-fold increase of the IgG level compared to the level on day 28.

To assess the Th1/Th2 bias in the IgG response elicited through immunization, we evaluated the IgG subtype profiles of our vaccine candidates (Figure 2D). Unexpectedly, the cytokine profile (IFN-γ/IL-4) of iNKT agonists is not correlated with the IgG2a/IgG1 antibody response. For example, although α-C-GC mainly stimulates the production of IFN-γ (Th1 cytokine), subtypes of anti-RBD antibodies were IgG1 with a low level of IgG2a, paralleling Wong and co-workers’ report on the vaccine adjuvanted by C34, a Th1-skewing iNKT agonist.

The adjuvant potency of αGC can be enhanced by appropriate formulation but very few studies have been devoted to examining the impact of formulation on the humoral response induced by Th1- and Th2-biasing agonists. As a result, the dimethyl sulfoxide (DMSO)-solubilized αGC,
α-GC, OCH, or C20:2 in the phosphate-buffered saline (PBS) solution were prepared and admixed with the RBD-Fc protein, respectively. Compared to the solution form, the liposomal formulation improves the adjuvant activity of all four iNKT agonists by stimulating a higher level of the anti-RBD IgG response on day 42. The increase fold is 2.0, 1.6, 3.0, and 1.4 for α-GC, α-C-GC, OCH, and C20:2, respectively, and a significant difference was observed for α-C-GC (Figure 2C).

Remarkably, α-GC significantly elicited a higher IgG2a titer in liposomes than in solution (Figure 2D).

**CONCLUSIONS**

Currently, the development of safe and effective vaccines is given a high priority. Here, we synthesized iNKT glycolipid agonists (α-GC, α-C-GC, OCH, or C20:2) through a universal synthetic route involving the construction of three contiguous
Figure 2. RBD-Fc adjuvanted by Th2-skewing agonists elicits a stronger neutralizing response than those by αGC and α-C-GC. Female BALB/c mice (n = 5 per group) were subcutaneously immunized on days 1, 15, and 29 with 2 μg of the RBD-Fc protein admixed with 2 μg of αGC or equal mols of other glycolipids (α-C-GC, OCH, or C20:2) as either a liposomal or solution form. Control mice were dosed with 2 μg of the RBD-Fc protein admixed with 100 μL of Alum. Antibody responses specific to RBD were assessed in antisera from immunized mice by the ELISA or pseudovirus neutralization assay. The data are indicated as the average ± SEM; each symbol represents one mouse serum. (A, B) IC50 titer of spike-pseudo-typed virus neutralization on day 42. The horizontal dashed line indicates the average neutralizing antibody titer of antisera immunized with 2 μg of RBD-Fc admixed with Alum on day 42. The deeper red color in the table represents a higher dilution ratio. (C) Anti-RBD IgG endpoint titers on days 14, 28, and 42. The horizontal dashed line indicates the average anti-RBD IgG titer of antisera immunized with 2 μg of RBD-Fc admixed with Alum on day 42. Asterisks without brackets indicate a significant difference to control RBD-Fc/Alum on day 42. (D) Anti-RBD IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3) on day 42. Asterisks without brackets indicate a significant difference from the corresponding IgG subclass titer elicited by RBD-Fc/Alum on day 42. The antibody titer below the limit of detection was set to 1. Asterisks without brackets indicate the significance of multiple groups in comparison to the control group evaluated using one-way ANOVA followed by Dunnett’s multiple comparison test. Pound signs with brackets indicate the significant difference calculated using unpaired two-tailed Student’s t-test. ns: not significant.
stereocenters in the phosphatidyl glycerol chain. The stereo-configuration and regiochemistry in the step of epoxide opening were confirmed. The low regioselectivity in the O-glycosides compared to the C-glycoside could be rationalized by the electron-withdrawing inductive effect caused by glycosidic oxygen which has been investigated by Sharpless and Behrens; therefore, this parallel comparison may serve as a cautionary note for organic synthesis involving the ring opening of 2,3-epoxy alcohols under the condition of NH$_2$Cl/NaNO$_3$.

Next, we demonstrated that αGC-adjuvanted RBD-Fc of high dose (32 μg) induced a significantly stronger humoral response, particularly neutralizing antibodies, than that observed with Alum. In the low dose (2 μg), the Th2-biasing glycolipids stimulated the most robust neutralizing antibody responses among the four evaluated iNKT agonists, significantly outperforming Alum. The potent adjuvant activity of OCH and C20:2 exhibited here is in line with the report by Kang and co-workers, which may be explained by their improved solubility over αGC. Of note, the antibody response induced by α-C-GC is statistically equal to the other glycolipid agonists, consistent with literature precedents in SARS-CoV-2 infections.

Given a discordance between virus-specific antibody levels and T-cell responses as well as an effective antitumor T-cell immunity triggered by α-C-GC, the Th1-biasing agonist could be a potent adjuvant for T-cell responses in COVID-19 vaccines. The studies of the cellular immune response of iNKT cell agonists are in progress.

In addition to enhancing the immunogenicity of the RBD-Fc protein, αGC-based glycolipids provide practical advantages in terms of the COVID-19 pandemic, as it enables a significant reduction in the antigen dose. Considering the facile manufacturing of the recombinant RBD-Fc protein and αGC-based glycolipids on a large scale, as well as the safety profile of αGC demonstrated in clinical trials, our data support the continued development of RBD-Fc formulated with the iNKT glycolipid agonist as a candidate vaccine to prevent the COVID-19 disease.

**EXPERIMENTAL SECTION**

**Chemistry. General Information.** All reactions were carried out under a dry Ar atmosphere using oven-dried glassware and magnetic stirring. The solvents were dried before use as follows: toluene, tetrahydrofuran (THF), and Et$_2$O were heated at the reflux over sodium; dichloromethane (DCM) was dried over CaH$_2$. Anhydrous N,N-diisopropylethylamine (DIPEA) and triethylamine were used directly as purchased. Commercially available reagents were used without further purification unless otherwise noted. Reactions were monitored by analytical TLC on silica gel 60 × 10$^{-2}$ mm glass plates. The spots were visualized with short wavelength UV light or by charring after spraying with a solution prepared from the following solutions: phosphomolybdic acid (5.0 g) in 95% EtOH (100 mL); p-anisaldehyde solution (2.5 mL of p-anisaldehyde, 2 mL of AcOH, and 3.5 mL of concentrated H$_2$SO$_4$ in 100 mL of 95% EtOH); or ninhydrin solution (0.3 g of ninhydrin was dissolved in 100 mL of n-butanol and 3 mL of AcOH was added). Flash chromatography was carried out with silica gel 60 (230–400 ASTM mesh). NMR spectra were obtained on a 400 or 600 MHz spectrometer. Proton chemical data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant, and integration. Chemical shifts were referenced to residual solvent peaks: CDCl$_3$ (δ = 7.26 ppm for $^1$H NMR and 77.00 ppm for $^{13}$C NMR), CD$_2$OD (δ = 3.31 ppm for $^1$H NMR and 49.00 ppm for $^{13}$C NMR). Electrospray ionization (ESI) mass was obtained on a Thermo Scientific Ultimate 3000/TSQ Quantum access MAX. The high-performance liquid chromatography system (HPLC) employed was an Agilent 1260 fitted with an evaporative light scattering detector (ELSD) detector. The purities of αGC, α-C-GC, OCH, and C20:2 are >95%, as determined by HPLC-ELSD (see the Supporting Information).

(1-But-3-en-1-yl)-2,3-bis-O-(benzyl)-4,6-O-(phylmethylene)-α-C-D-galactopyranoside (8). To the solution of S (3.2 g, 6.93 mmol) in anhydrous THF (70 mL) was added NaN$_3$ (5.7 g, 0.06 M solution in THF, 27.7 mL, 13.86 mmol) at rt. After 3 h of stirring at rt, EtOH (28.0 mL), H$_2$O (38 mL, 296.59 mmol), and 30% aqueous solution of NaOH (9.3 mL) were added at 0°C, and the resulting mixture was stirred at rt for 1 h, then treated with 100 mL saturated aqueous NaCl solution. The organic layer was separated, and the aqueous layer was extracted with Et$_2$O. The combined organic phase was dried (MgSO$_4$) and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (petroleum ether/ethyl acetate = 1:1) on silica gel to provide the primary alcohol (3.24 g, 95%) as colorless oil. GC demonstrated in clinical trials, our data support the continued development of RBD-Fc formulated with the iNKT glycolipid agonist as a candidate vaccine to prevent the COVID-19 disease.
(1.50 g, 3.09 mmol) and 7 (1.83 g, 6.18 mmol) in CH₂Cl₂ (30 mL) was added Grubs second generation catalyst (394 mg, 0.46 mmol, 15 mol%). The reaction mixture was stirred for 2 h at reflux under Ar and then reduced in volume to 1 mL. Purification by flash column chromatography on silica gel (petroleum ether/ethyl acetate = 8:1) provided protected-acetate alcohol (1.91 g, 82%) as a white solid. 1H NMR (400 MHz, CDCl₃): δ 7.54 (d, J = 7.0 Hz, 2H), 7.46–7.14 (m, 13H), 5.49 (s, 1H), 4.93–4.52 (m, 4H), 4.22 (m, 4H), 4.01 (d, J = 12.7 Hz, 1H), 3.74 (d, J = 12.1 Hz, 1H), 3.61 (s, 1H), 3.46 (s, 1H), 1.96 (s, 2H), 1.77–1.39 (m, 2H), 1.26 (s, 24H), 0.88 (t, J = 6.6 Hz, 3H). 13C NMR (100 MHz, CDCl₃) 138.5, 138.7, 128.9, 128.5, 128.1, 127.8, 127.7, 127.6, 126.3, 107.9, 101.1, 75.8, 75.6, 74.5, 73.6, 72.1, 71.6, 69.9, 63.1, 31.9, 31.4, 29.7, 29.6, 29.6, 29.3, 26.7, 25.6, 22.7, 20.5, 14.1. MS (ESI): calcd for C₄₉H₆₉NaO₇+ [M + Na]+, 843.5033; found, 843.5021.

(3,5,5′,5″)-1-(3′-Azido-4′,5′,5″-dimethyl-1,3-dioxolane)-octadecyl-2,4,8,12-tetramethyl-3,7-trioxa-tricosanoyl acid (11). To a solution of diol 10 (545 mg, 0.706 mmol) in anhydrous MeOH (6 mL) was added 2,2-DMP (86 μL, 0.7 mmol) followed by PPTS (18 mg, 0.0706 mmol). After the reaction mixture was stirred at rt for 1.5 h, the saturated NaHCO₃ (10 mL) was added, and the mixture was extracted with CH₂Cl₂ (3 × 20 mL). The combined organic phases were dried over Na₂SO₄. After evaporation of the solvent under reduced pressure, the residue was purified by flash column chromatography (petroleum ether/ethyl acetate = 9:1) to give product 11 (430.5 mg, 75%) as a colorless solid. 1H NMR (400 MHz, CDCl₃): δ 7.55 (d, J = 6.8 Hz, 2H), 7.45–7.10 (m, 13H), 5.50 (d, J = 2.5 Hz, 1H), 4.93–4.56 (m, 4H), 4.21 (q, J = 11.0, 6.6 Hz, 4H), 4.12 (s, 1H), 4.01 (d, J = 12.6 Hz, 2H), 3.87 (t, J = 7.5 Hz, 1H), 3.74 (d, J = 9.2 Hz, 1H), 3.45 (d, J = 13.8 Hz, 2H), 1.99 (d, J = 12.0 Hz, 2H), 1.70 (s, 2H), 1.51 (s, 4H), 1.39 (d, J = 2.5 Hz, 3H), 1.32 (d, J = 2.4 Hz, 3H), 1.26 (d, J = 2.5 Hz, 24H), 0.88 (s, 3H). 13C NMR (100 MHz, CDCl₃): δ 138.7, 138.6, 137.8, 138.3, 128.3, 128.1, 127.6, 127.5, 127.5, 126.3, 108.1, 101.2, 78.0, 77.8, 76.6, 75.6, 75.6, 74.6, 73.6, 71.6, 69.9, 63.1, 60.1, 31.9, 29.7, 29.6, 29.6, 29.3, 26.1, 26.3, 25.7, 22.7, 19.5, 14.1. MS (ESI): calcd for C₄₉H₆₉NaO₇+ [M + Na]+, 834.5033; found, 834.5021.

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Concentration under reduced pressure. The resulting residue was filtrated through a pressed pad of Celite, then the filtrate was quenched by the saturated NaHCO_{3} solution, and neutralized with NaHCO_{3} aqueous solution. The biphasic mixture was separated, and the aqueous layers were extracted with CHCl₃. The organic layer was dried (Na₂SO₄) and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate = 2:1) on silica gel, which was prewashed with CH₂Cl₂/MeOH (1:1) to provide the cross-metathesis product.

A solution of the activated 4 Å molecular sieves (317.8 mg, n-DIP) in DCM (17 μL) was added dropwise, and the mixture was stirred at 90 °C for 3 h. The resulting mixture was filtered through a pressed pad of Celite, and the filtrate was washed through a pressed pad of Celite, then the filtrate was quenched by the saturated NaHCO₃ solution, and neutralized with NaHCO₃ aqueous solution. The biphasic mixture was separated, and the aqueous layers were extracted with CHCl₃. The organic layer was dried (Na₂SO₄) and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate = 2:1) on silica gel, which was prewashed with CH₂Cl₂/MeOH (1:1) to provide the cross-metathesis product.

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3.83–3.70 (m, 2H), 3.69–3.55 (m, 2H), 3.44 (m, 1H), 1.51 (m, 2H), 1.29 (m, 24H), 0.90 (t, J = 6.9 Hz, 3H). 13C NMR (150 MHz, CDCl3): δ 138.2, 137.8, 137.6, 128.9, 128.5, 128.3, 128.3, 128.2, 128.1, 128.0, 127.8, 127.6, 127.2, 101.0, 99.7, 79.8, 79.1, 75.4, 74.4, 73.8, 73.3, 71.9, 69.4, 68.2, 65.9, 62.0, 50.3, 36.7, 31.9, 30.5, 30.2, 29.7, 29.7, 29.6, 29.4, 29.3, 25.7, 22.9, 14.1. MS (ESI): calcd for C16H18N3O6 [M + Na]+, 290.2547; found, 290.2568.

(2S,4aR,5R,7R,8S,8aS)-6-(((2S,3S,4R)-2-Azido-3,4-bis(benzyloxy)-nonyl)oxy)-7,8-bis(benzyloxy)-2-phenylhexahydropyrano[3,2-d]-1,3-dioxine (19-αGC). This compound was prepared from 19-αGC in 81% yield by the procedure described above (19-OCH to 20-OCH). 1H NMR (600 MHz, CDCl3): δ 7.62–7.16 (m, 25H), 5.79 (d, J = 8.2 Hz, 1H), 5.48 (s, 1H), 4.96 (d, J = 3.5 Hz, 1H), 4.91–4.46 (m, 8H), 4.37–3.46 (m, 11H), 1.87 (m, 2H), 1.73–1.59 (m, 3H), 1.53–1.45 (m, 2H), 1.46–1.09 (m, 67H), 0.90 (t, J = 6.9 Hz, 6H). 13C NMR (151 MHz, CDCl3): δ 172.9, 138.5, 138.8, 128.4, 128.3, 128.1, 127.9, 127.8, 127.7, 127.6, 127.5, 126.3, 101.0, 99.7, 79.8, 76.9, 75.7, 74.4, 73.8, 71.9, 69.4, 62.9, 36.7, 31.9, 29.7, 29.6, 29.5, 29.4, 25.7, 22.7, 14.1. MS (ESI): calcd for C40H38NNaO9 [M + Na]+, 792.6043; found, 792.6047.

13C NMR (600 MHz, CDCl3): δ 7.57–7.18 (m, 25H), 5.45 (s, 1H), 4.97 (d, J = 3.5 Hz, 1H), 4.88–4.47 (m, 8H), 4.22–3.97 (m, 5H), 3.87 (d, J = 12.5 Hz, 1H), 3.78–3.53 (m, 3H), 1.66 (m, 7.6 Hz, 1H), 1.53 (m, 1H), 1.47–1.38 (m, 1H), 1.27 (m, 2H), 0.88 (t, J = 7.1 Hz, 3H). 13C NMR (150 MHz, CDCl3): δ 138.7, 138.3, 137.9, 137.8, 128.4, 128.3, 128.2, 128.2, 128.1, 127.9, 127.8, 127.7, 127.6, 127.6, 127.4, 126.1, 101.0, 99.1, 79.4, 78.8, 73.7, 73.5, 72.0, 31.9, 22.6. MS (ESI): calcd for C16H16N4O4 Na+ [M + Na]+, 393.3546; found, 393.3493.

30.5, 30.3, 30.0, 30.0, 26.6, 26.1, 23.3, 14.3. MS (ESI): calcd for C40H38NNaO9 [M + Na]+, 792.6043; found, 792.6047.

13C NMR (150 MHz, CDCl3): δ 7.57–7.19 (m, 25H), 5.45 (s, 1H), 4.97 (d, J = 3.5 Hz, 1H), 4.88–4.47 (m, 8H), 4.22–3.97 (m, 5H), 3.87 (d, J = 12.5 Hz, 1H), 3.78–3.53 (m, 3H), 1.66 (m, 7.6 Hz, 1H), 1.53 (m, 1H), 1.47–1.38 (m, 1H), 1.27 (m, 2H), 0.88 (t, J = 7.1 Hz, 3H). 13C NMR (150 MHz, CDCl3): δ 138.7, 138.3, 137.9, 137.7, 128.8, 128.3, 128.2, 128.2, 128.1, 127.9, 127.7, 127.6, 127.6, 127.4, 126.3, 101.0, 99.1, 79.3, 78.8, 73.5, 73.4, 72.0, 69.3, 68.4, 62.9, 61.7, 31.9, 31.6, 29.9, 29.7, 29.3, 25.4, 22.7, 14.1. MS (ESI): calcd for C16H16N4O4 Na+ [M + Na]+, 393.3546; found, 393.3493.

13C NMR (150 MHz, CDCl3): δ 7.57–7.19 (m, 25H), 5.45 (s, 1H), 4.97 (d, J = 3.5 Hz, 1H), 4.88–4.47 (m, 8H), 4.22–3.97 (m, 5H), 3.87 (d, J = 12.5 Hz, 1H), 3.78–3.53 (m, 3H), 1.66 (m, 7.6 Hz, 1H), 1.53 (m, 1H), 1.47–1.38 (m, 1H), 1.27 (m, 2H), 0.88 (t, J = 7.1 Hz, 3H). 13C NMR (150 MHz, CDCl3): δ 138.7, 138.3, 137.9, 137.7, 128.8, 128.3, 128.2, 128.2, 128.1, 127.9, 127.7, 127.6, 127.6, 127.4, 126.3, 101.0, 99.1, 79.3, 78.8, 73.5, 73.4, 72.0, 69.3, 68.4, 62.9, 61.7, 31.9, 31.6, 29.9, 29.7, 29.3, 25.4, 22.7, 14.1. MS (ESI): calcd for C16H16N4O4 Na+ [M + Na]+, 393.3546; found, 393.3493.
**Dioxolane 25 and Dioxane 26.** To a solution of compound 17-OCH (64.3 mg, 0.099 mmol) in anhydrous DCM (5 mL) were added PPTS (2.5 mg, 0.010 mmol) and 2,2-DMP (124.5 µL, 0.992 mmol), and the solution was stirred at rt overnight. The mixture was concentrated under reduced pressure. The resulting residue was purified through flash column chromatography on silica gel (petroleum ether/ethyl acetate = 9:1) to provide compound 25 (50.5 mg, 74%) as a white solid. Compound 26 was synthesized using the same method from 18-OCH (72%). NMR data for 25: 1H NMR (600 MHz, CDCl3; δ 7.52 (d, J = 7.2 Hz), 7.34 (m, 13H), 5.48 (s, 1H), 5.03 (d, J = 3.3 Hz), 1H), 4.77 (m, 4H), 4.27–4.15 (m, 2H), 4.15–3.98 (m, 6H), 3.77 (m, 1H), 3.36 (s, 1H), 3.38 (t, J = 7.4 Hz, 1H), 1.45–1.24 (m, 12H), 0.91 (d, J = 6.6 Hz, 3H). 13C NMR (150 MHz, CDCl3; δ 138.9, 138.8, 137.8, 128.9, 128.2, 128.1, 127.7, 127.6, 127.5, 127.4, 126.3, 126.3, 126.2, 125.8, 124.7, 124.6, 124.5, 123.9, 123.8, 122.5, 122.4, 120.1, 101.1, 99.4, 77.7, 75.6, 75.0, 74.9, 73.2, 72.3, 69.5, 69.4, 63.1, 59.7, 31.8, 29.3, 28.2, 26.1, 25.7, 22.6, 14.0. 26: 1H NMR (600 MHz, CDCl3; δ 7.52 (d, J = 6.9 Hz, 2H), 7.46–7.24 (m, 10H), 5.49 (s, 1H), 5.09 (d, J = 3.6 Hz, 1H), 4.79 (m, 4H), 4.27–4.16 (m, 2H), 4.13–3.97 (m, 3H), 3.75 (m, 5H), 3.43 (m, 1H), 1.66–1.55 (m, 2H), 1.48–1.12 (m, 12H), 0.90 (t, J = 6.7 Hz, 3H). 13C NMR (150 MHz, CDCl3; δ 138.7, 137.8, 128.8, 128.5, 128.2, 128.1, 127.7, 127.5, 127.3, 126.3, 101.1, 101.0, 98.2, 75.6, 75.4, 74.7, 73.2, 72.1, 71.5, 70.3, 69.4, 69.6, 62.8, 62.6, 31.7, 29.9, 25.3, 24.4, 23.9, 22.5, 14.0.

**Immunological Test. Materials and Reagents.** DSPC was purchased from TCI. Cholesterol was purchased from Energy Chemical. Peroxidase-conjugated AffiniPure goat anti-mouse kappa, IgA, IgE, IgG1, IgG2a, IgG2b, and IgG3 antibodies were purchased from Southern Biotechnology, peroxidase-conjugated AffiniPure goat anti-mouse κ antibodies IgG and IgM were purchased from Jackson Immuno Research. RBD-Fc (catalogue number: 40592-V08B), and RBD-His (catalogue number: 40592-V02H), and RBD-His (Sino Biological), which had been dissolved in 0.1 M PBS (pH = 7.4), were purchased from Sino Biological, Beijing, China. The lentiviral packaging plasmid psPAX2 and the SARS-CoV-2-S was kindly provided by Prof. Xingyi Ge (Hunan University, China). The lentiviral packaging plasmid psPAX2 and the pLenti-GFP reporter plasmid expressing both GFP and luciferase were purchased from Addgene (Cambridge, MA).

**SARS-CoV-2 Pseudovirus Production and Titration.** For the preparation of the SARS-CoV-2 pseudovirus, psPAX2, pLenti-GFP, and pCDNA3.1-SARS-CoV-2-S was kindly provided by Prof. Xingyi Ge (Hunan University, China). The lentiviral packaging plasmid psPAX2 and the pLenti-GFP reporter plasmid expressing both GFP and luciferase were purchased from Addgene (Cambridge, MA).

**SARS-CoV-2 Pseudovirus Neutralization Assay.** Neutralizing antibodies in mice were assessed by the SARS-CoV-2-pseudo-type neutralization assay as described previously. The HeLa-ACE2 cells were seeded into 96-well plates and incubated with 50 µL of media containing pseudovirions. After 8 h incubation, the supernatant was replaced with fresh DMEM containing 2% FBS. At 48 h, postincubation, the cells were lysed with 30 µL of lysis buffer (Promega, Madison, WI, USA), and the transduction efficiency was measured by the quantification of the luciferase activity using the Luciferase Assay Kit (Promega) according to the manufacturer’s instructions. All the experiments were done in triplicates and repeated at least twice.

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titers (IC₅₀) were calculated as the reciprocal of the serum dilution that resulted in a 50% reduction in RLUs compared to virus control wells after the subtraction of background RLU. The 1:100 serum dilution was the limit of quantitation in this assay. Serum samples that did not exhibit neutralizing activities or that neutralized at levels higher than 1:100 were calculated as the limit of the quantification for statistical analyses. IC₅₀ below the limit of detection was determined as half the limit of detection.

**Statistical Analysis.** Data reported in the figures were analyzed using Prism 6 (GraphPad Software). All values and error bars are mean ± SEM. The significance between the two groups was determined by the unpaired two-tailed Student’s t-test. The significance of multiple groups in comparison to the control group was evaluated using one-way ANOVA followed by Dunnett’s multiple comparison test. Asterisks (*, P ≤ 0.05; **, P ≤ 0.01; ###, P ≤ 0.001; ****, P ≤ 0.001; ns, not significant) indicate significant differences.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00881.

Compound characterization spectra (HPLC-ELSD, ¹H NMR, and ¹³C NMR), vaccine liposomal formulations, information of RBD-Fc and RBD-His, and biological assays (IgM, IgG subtype response induced by a different dose of RBD-Fc) (PDF)

Molecular formula strings of the compounds (CSV)

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**Notes**
The authors declare no competing financial interest.

**ABBREVIATIONS**
αGC, α-galactosylceramide; ACE2, angiotensin-converting enzyme 2; APC, antigen-presenting cells; 9-BBN, 9-borabicyclo[3.3.1]nonane; BSA, bovine serum albumin; DCS, dendritic cells; DCM, dichloromethane; DIPEA, N,N-diisopropylethylamine; 2,2-DMP, 2,2-dimethoxypropane; DMEM, Dulbecco’s modified Eagle’s medium; DSPC, 1,2-distearoyl-sn-glycerol-3-phosphocholine; ELISA, enzyme-linked immunosorbent assay; ELSD, evaporative light scattering detector; ESI, electrospray ionization; FBS, fetal bovine serum; HRP, horseradish peroxidase; IC₅₀,half-maximal inhibitory concentration; IFN-γ, interferon-γ; IL-4, interleukin-4; iNKT, invariant natural killer T; MHC, major histocompatibility complex; MTPA, methoxy-α-(trifluoromethyl)phenylacetic acid; PPTS, pyridinium 4-
toluenesulfonate; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TLC, thin-layer chromatography

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