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A Rapid Fluorescence-Based Assay for Classification of iNKT Cell Activating Glycolipids

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Supporting Information

ABSTRACT: Structural variants of α-galactosylceramide (αGC) that activate invariant natural killer T cells (iNKT cells) are being developed as potential immunomodulatory agents for a variety of applications. Identification of specific forms of these glycolipids that bias responses to favor production of proinflammatory vs anti-inflammatory cytokines is central to current efforts, but this goal has been hampered by the lack of in vitro screening assays that reliably predict the in vivo biological activity of these compounds. Here we describe a fluorescence-based assay to identify functionally distinct αGC analogues. Our assay is based on recent findings showing that presentation of glycolipid antigens by CD1d molecules localized to plasma membrane detergent-resistant microdomains (lipid rafts) is correlated with induction of interferon-γ secretion and Th1-biased cytokine responses. Using an assay that measures lipid raft residency of CD1d molecules loaded with αGC, we screened a library of ∼200 synthetic αGC analogues and identified 19 agonists with potential Th1-biasing activity. Analysis of a subset of these novel candidate Th1 type agonists in vivo in mice confirmed their ability to induce systemic cytokine responses consistent with a Th1 type bias. These results demonstrate the predictive value of this novel in vitro assay for assessing the in vivo functionality of glycolipid agonists and provide the basis for a relatively simple high-throughput assay for identification and functional classification of iNKT cell activating glycolipids.

Invariant natural killer T cells (iNKT cells) constitute a unique lymphocyte subset that participates in immune responses against a wide variety of infectious diseases and tumors and also plays a role in regulating allergic and autoimmune diseases.[1,2] These cells express surface markers associated with both conventional thymus-derived (T) cells and natural killer (NK) cells. They also express an invariant α chain as a component of their T cell antigen receptors (TCRs).[1] The TCRs of these cells are specific for a range of different glycolipid antigens, which are recognized as noncovalent complexes with CD1d, a cell surface glycoprotein that is homologous to class I antigen presenting molecules encoded by the major histocompatibility complex (MHC).[3] The prototype glycolipid antigen of mouse and human iNKT cells is a form of α-galactosylceramide (αGC) designated as KRN7000 (**1**).[4] Recognition of this glycolipid is highly conserved between mouse and human, although studies suggest subtle differences in responses by iNKT cells in these species.[5] The capability of iNKT cells to activate or regulate NK cells, dendritic cells (DCs), neutrophils, macrophages, and B and conventional T cells makes them an attractive target for immunotherapeutics. Indeed, activation of iNKT cells with KRN7000 has been shown to augment beneficial host immunity in some models and to suppress autoimmune diseases in others.[2,6]

Administration of **1** to mice induces a mixed cytokine response that is characterized by secretion of both IFNγ and IL-4, the prototypical cytokines defining anti-inflammatory (Th2) versus proinflammatory (Th1) cellular immune responses.[7] The agonist **1** is therefore classified as inducing a nonpolarized or Th0 response. The secretion of IL-4 peaks at 2 h post-injection, while IFNγ secretion peaks at ∼12 h and is sustained for up to 36 h after glycolipid administration. The majority of this systemic IFNγ secretion does not originate from the iNKT cells but is the result of secondary activation of NK cells.[8] Variants of αGC with truncated or unsaturated acyl chains (e.g., αGC C20:2; compound **2** in Figure 1) can bias the response to favor production of Th2-type cytokines.[3,8−10] This is characterized by strong IL-4 secretion with reduced IFNγ production due to the failure to transactivate NK cells. Such Th2-biased iNKT cell activators have been found to be superior for treatment in several mouse models of autoimmune disease.[7,10] On the contrary, iNKT cell agonists that bias responses to stronger and more sustained production of Th1-type cytokines are more effective as adjuvants for inducing protective immunity against infections and for anticancer immunity. The most extensively studied Th1-biasing agonist is the C-glycoside of KRN7000, α-C-GC (**3**), in which the oxygen of the glycosidic bond has been replaced with a one-carbon linker.[3,11] This agonist induces

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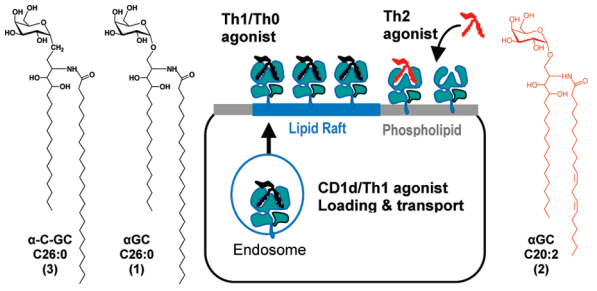

Figure 1. Prototype αGC activators of iNKT cells and a schematic summarizing pathways for their loading onto and presentation by CD1d.

minimal secretion of IL-4 and is associated with enhanced NK cell activation that results in higher and more sustained IFNγ secretion. Variants of this agonist containing an olefin linkage also display enhanced Th1 bias in cytokine responses.[12]

A commonly used screening assay to identify new αGC analogues is based on the culture of splenic cells in the presence of candidate agonists followed by quantification of cytokines in the supernatants and calculation of the ratio of IL-4 to IFNγ.[8,10,13] Many Th2-biasing iNKT agonists have been successfully identified in this way. Unfortunately, this type of screening assay has proved to be less effective for identifying Th1-biasing agonists. Generally, the prototypical Th1-biasing α-C-GC analogue **3** has been found to display little or no activity in such cell culture assays, despite the Th1-biased response that is observed in vivo following injection into mice.[14] At present, identification of strong IFNγ-inducing or Th1-biasing agonists is possible only by analyzing responses of intact animals following systemic injection of candidate agonists, an approach that is not amenable to high-throughput screening. This represents a major limitation in the identification of additional and potentially more potent IFNγ-inducing iNKT cell agonists. Thus, the development of a relatively simple and rapid in vitro assay that reliably predicts the Th1 versus Th2 functionality would be an important step toward accelerating the development of new and more effective iNKT cell agonists for a wide variety of potential clinical applications.

We recently showed that αGC agonists that induce strong IFNγ secretion from NK cells have differences in CD1d loading and presentation relative to Th2-biasing agonists.[13] The former agonists load CD1d in an endocytic compartment and are selectively trafficked to lipid raft microdomains in the plasma membrane. In contrast, Th2-biasing agonists are loaded directly into CD1d proteins present on the cell surface. Initial studies indicated that CD1d complexes formed with Th2-biasing agonists tend to be pH-labile and therefore dissociate at the low pH typical of endosomal compartments. Since endosomal trafficking is required for lipid raft localization, these complexes are excluded from lipid rafts (Figure 1). Localization in lipid rafts is probably responsible for optimal stimulation of iNKT cells, which ensures that transactivation of NK cells takes place. We therefore evaluated the possibility of using quantitation of lipid raft residency of CD1d/glycolipid complexes as a method of identifying new αGC agonists that have the potential to stimulate strong IFNγ production and Th1-biased cytokine responses. Here we describe the development of a rapid two-step flow cytometry-based assay employing this principle to identify potent αGC analogues and predict their functional properties.

To screen a large library of αGC analogues, we first selected αGC candidate agonists that showed efficient binding and presentation by CD1d. Individual glycolipids were incubated at 100 nM for 12 h with the $CD1d^+$ murine dendritic cell line JAWS II[15] followed by quantitation of the binding of monoclonal antibody (mAb) L363 to cell surface CD1d/glycolipid complexes by flow cytometry. This mAb is specific for complexes formed by the binding of αGC to murine CD1d.[16] Using this approach, we screened a library of 193 synthetic αGC analogues containing modifications of fatty acyl, sphingosine, and glycosidic linkages. This library, provided in Table S1 in the Supporting Information (SI), included a large diversity of fatty acyl modifications as well as all eight stereoisomers of KRN7000 and carbasugar replacement of the galactose residues. Several of these agonists have been tested in vitro and in vivo for their iNKT stimulatory activities (Table S2). In the initial stage of screening, we selected all of the agonists that gave an increase in L363 staining of at least 4 standard deviations above the median fluorescence intensity (MFI) of untreated control cells (Figures S1 and S2 in the SI). This represented a relatively stringent criterion for CD1d binding that actually excluded some of the known weak iNKT cell agonists (e.g., the weak Th2-biasing compound known as OCH).[7] This selection reduced the candidate agonists to 71, which were then investigated for lipid raft localization using the detergent extraction method.

For quantitation of the extent of lipid raft residency, we exploited the fact that lipid raft microdomains are resistant to extraction with nonionic detergents such as Triton X-100 (Tx-100). Thus, JAWS II cells were incubated with each αGC analogue at 100 nM concentration and stained with Alexa Fluor 647-conjugated mAb L363. Since plasma membrane lipid rafts are detergent-resistant, CD1d/glycolipid agonist complexes localized in lipid rafts cannot be extracted, and a minimal decrease in fluorescence intensity is observed over time. In contrast, for the CD1d/glycolipid agonist complexes that are excluded from lipid rafts, the MFI decreases sharply following Tx-100 addition. We also considered that a decrease in MFI following Tx-100 treatment could be caused by the dislodgement of fluorescently conjugated mAb from the CD1d/αGC complexes. This possibility was excluded by studies of the effect of Tx-100 concentration on the binding of L363 with its ligand, as we showed using a cell-free assay that the low concentration of Tx-100 (0.06%) used for this assay did not cause dissociation of L363 mAb from CD1d/αGC complexes (Figure S3). To perform the analysis of Tx-100 stability of cell surface CD1d/αGC complexes, the MFI of cells was first recorded for ~5 s to obtain a baseline starting value, and then Tx-100 was added to a final concentration of 0.06%. The sample was vortexed briefly to ensure uniform mixing, and data were acquired using flow cytometry in kinetic mode to visualize changes in MFI over time (Figure 2A). To validate the screening assay, we chose agonists **1**−**3** as representative Th0, Th2, and Th1 cytokine-biasing glycolipid agonists. CD1d complexes containing agonist **2** were rapidly eluted from the cell surface by Tx-100 (Figure 2), consistent with their exclusion from lipid rafts. This agonist induced high levels of IL-4 but failed to induce sustained IFNγ at the 24 h time point. In contrast, both **1** and **3** were presented by detergent-resistant, lipid-raft-resident CD1d and induced strong IFNγ responses in mice with 1−3 orders of magnitude greater Th1 bias than for **2**.

Since several known Th2-biasing αGC analogues in the library showed reductions of up to 70% or more in the initial fluorescence during a 30 s exposure to Tx-100, we classified agonists as negative for lipid raft localization if they showed

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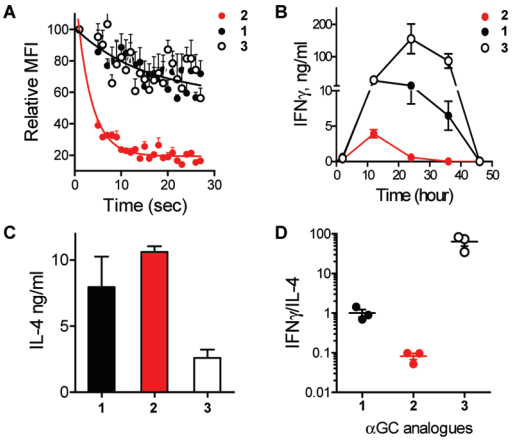
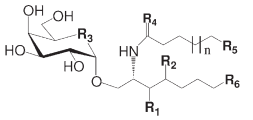


Figure 2. Validation of the rapid in vitro fluorescent antibody staining assay to predict the functional activity of αGC analogues. (A) Plot of detergent extraction from JAWS II dendritic cells of CD1d/glycolipid complexes formed with known Th1- and Th2-biasing αGC agonists (i.e., compounds **1**−**3** in Figure 1). (B) In vivo serum IFNγ levels at various time points following administration of 4 nmol of αGC analogues. (C) Serum IL-4 secretion measured 2 h after injection of αGC analogues. (D) Ratio of the IFNγ level observed at 24 h with that of peak IL-4 secretion 2 h after glycolipid administration.



	Name	n	R_1	R_2	R_3	R_4	R_5	R_6
4	SKC08-20	2	OH	H	O	O	Phe	$C_{11}H_{23}$
5	SKC08-19	21	OH	H	O	O	CH_3	$C_{11}H_{23}$
6	SKC08-22a	21	H	H	O	O	CH_3	$C_{11}H_{23}$
7	SKC08-22b	21	OH	H	O	O	CH_3	$C_{11}H_{23}$
8	SKC08-27	21	OH	OH	CH_2	O	CH_3	$C_{11}H_{23}$
9	SKC08-29	21	H	H	CH_2	O	CH_3	$C_{11}H_{23}$
10	SKC08-30	21	OH	H	CH_2	O	CH_3	$C_{11}H_{23}$
11	SKC08-31	21	OH	H	CH_2	O	CH_3	$C_{11}H_{23}$
12	DB06-1	21	OH	OH	O	S	CH_3	$C_{11}H_{23}$
13	DB03-1	15	OH	OH	O	O	CH_3	$C_{11}H_{23}$
14	DB01-1	19	OH	OH	O	O	CH_3	$C_{11}H_{23}$
15	DB04-2	23	OH	OH	O	O	CH_3	$C_{11}H_{23}$
16	SKC08-25	21	OH	OH	CH_2	O	CH_3	Phe
17	SKC08-26	21	OH	OH	CH_2	O	CH_3	Phe
18	SKC07-1	21	OH	OH	O	O	CH_3	$C_{11}H_{23}$
19	SKC07-2	21	OH	OH	O	O	CH_3	$C_{11}H_{23}$
20	SKC07-4	21	OH	OH	O	O	CH_3	$C_{11}H_{23}$
21	YTC3-37	11	OH	OH	O	O	CH_3	$C_{11}H_{23}$

Figure 3. Structures of predicted cytokine-biasing agonists. **4** was presented by Tx-100-extractable CD1d molecules and thus predicted to be a Th2-biasing agonist. In contrast, agonists **5**−**21** were presented by detergent-resistant (i.e., raft-localized) CD1d and were therefore identified as potential strong IFNγ inducers and Th1-biasing agonists.

more than 50% reduction in MFI within 30 s after addition of Tx100. Out of the 71 compounds analyzed, 51 were rapidly extracted with Tx100, consistent with their being bound predominantly to CD1d proteins localized outside of lipid rafts (Figure S4A,B). Of these 51 agonists, four had been previously studied for biological activity in vivo by injection into mice,[7,10] In all cases Th2-biased serum cytokine responses were elicited with high secretion of IL-4 relative to IFNγ (Table S2). These findings were consistent with the predictions of our lipid raft presentation model and indicated that our assay was able to accurately identify Th2-type iNKT cell agonists. In addition, we identified 17 new agonists in our library that formed CD1d complexes that were resistant to extraction with detergent (Figure 3 and Figures S4C and S5) and were thus predicted to induce strong and sustained IFNγ secretion following glycolipid administration in vivo.

To test this prediction, we chose a random sample of seven agonists (**5**, **6**, **8**, **9**, **10**, **12**, and **16**) from this group and tested their activities in vivo by injecting them individually into mice (4 nmol per mouse) (Figure 4). One of the predicted novel Th2-biasing agonists (**4**) was included as a reference standard. **4** induced relatively low IFNγ at the 2 h time point, and this was undetectable by the 24 h time point. In contrast, all seven compounds that were presented by non-Tx-100-extractable CD1d molecules induced significantly higher levels of IFNγ (Figure 4A) and sustained measurable serum levels of this cytokine for extended time periods of up to 36 h (Figure S6).

A useful indicator of Th1 bias in the cytokine response is the ratio of IFNγ observed 24 h after glycolipid injection to that for peak IL-4 secretion at 2 h. This ratio has typically been found to be 0.1−0.2 for well-characterized Th2-biasing agonists such as **2** (Figure 2D). In contrast, the prototypical agonist **1** gives a ratio of ∼1, while the strongly Th1-biasing agonist **3** induces very high IFNγ with very low IL-4 secretion, resulting in ∼**2** fold increase in this ratio (Figure 2D). The 4-dehydroxy-sphingoid base variant of **2** (agonist **5**) behaved like a strongly Th1-biasing agonist in vivo in mice, showing the highest secretion of IFNγ at 24 h with minimal IL-4 induction relative to all of the other agonists tested (Figure 4B).

Our data also showed that the extent of localization of CD1d/αGC complexes into lipid rafts was directly related to the fatty acyl chain length of the glycolipid. This was readily apparent from a comparison of the extents of lipid raft localization for six different agonists from our library that differed only in their fatty acyl chain lengths (Figure 5A). All of the agonists containing saturated fatty acyl moieties with < 16 carbons were excluded from lipid rafts, whereas all of the agonists containing saturated acyl chains with 16−26 carbons showed enhanced localization in lipid rafts. Introduction of diunsaturation in the C20 acyl chain abolished lipid raft localization of CD1d-loaded complexes for agonist **2**, suggesting that the hydrophobicity of the acyl chain rather than simply the chain length is the feature that determines the extent of trafficking and localization of CD1d/αGC complexes to lipid rafts. Further increasing the hydrophobicity of **1** by replacing the glycosidic linkage with CH_2 (as in the case of **3**), replacing the carbonyl oxygen with a sulfur atom (agonist **12**), creating deoxy versions (agonists **5**, **6**, **9**, and **10**), or replacing the galactose sugar with a structurally analogous carbasugar (agonists **9**−**11**) did not significantly increase lipid raft localization relative to **1** (Figure 5B). Furthermore, the carbasugar variant of **2** was still lipid-raft-excluded and showed a Th2-biased cytokine response in vivo (data not shown). Importantly, the screening assay described here is to our knowledge the only one to date that has been demonstrated to correctly predict the Th1-biasing ability of **3** using an in vitro method. However, given the similar levels of raft localization for all Th1/Th0-biasing glycolipids, the very pronounced IFNγ responses and Th1 biases of compounds **3** and **5** cannot be explained solely by enhanced lipid raft association. The other factors that further augment the Th1 bias for these compounds remain to be established.

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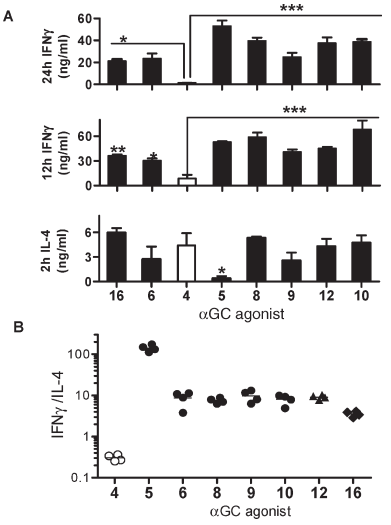

Figure 4. In vivo serum cytokine responses of selected αGC agonists. (A) Serum IL-4 and IFNγ at the indicated times after glycolipid injection were analyzed by ELISA. Results for **4** were compared with those for the seven other agonists using one-way analysis of variance (ANOVA) with Dunnet correction. *, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively. (B) Ratios of IFNγ observed 24 h after glycolipid injection to that for peak IL-4 at the 2 h time point.

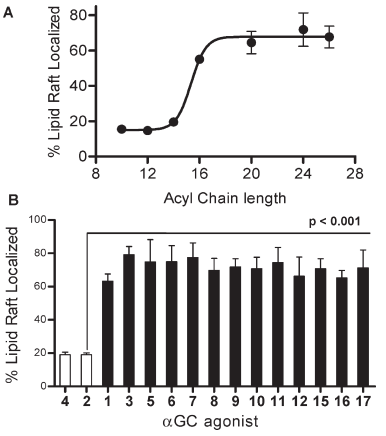

Figure 5. (A) Effect of saturated fatty acyl chain length on the extent of lipid raft localization. The percent lipid raft localization is plotted against the fatty acyl chain length of the αGC agonist. This shows a switch from lipid raft exclusion to lipid raft localization at a chain length of 16 carbons. (B) Comparison of the extent of lipid raft localization of CD1d loaded with 13 different Th1/Th0-biasing agonists vs Th2-biasing agonists **2** and **4**. The value for **2** was compared with the values for the other agonists using one-way ANOVA with Dunnet correction.

In summary, we have described a new in vitro method for rapid screening of large libraries of αGC analogues to predict their in vivo functionality. Our technique is simple and robust, and its use can substantially reduce the time and materials required for initial characterization of biological activity. The use of this straightforward in vitro approach should further advance our understanding of structure−activity relationships for αGC analogues and thus facilitate the discovery of iNKT cell agonists for a wide variety of immunotherapeutic applications. Our findings also provide further support for the importance of lipid raft localization of CD1d/αGC complexes as one of the significant factors that tunes the iNKT cell response. Further studies are underway to explore whether the approach described here can be applied to predict functional responses induced by glycolipid antigens in humans.

■ ASSOCIATED CONTENT

Supporting Information. Complete ref 13 and supplementary information. This material is available free of charge via the Internet at http://pubs.acs.org.

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