Structural and Functional Characterization of a Novel Nonglycosidic Type I NKT Agonist with Immunomodulatory Properties


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Activation of type I NKT (iNKT) cells by CD1d-presented agonists is a potent immunotherapeutic tool. α-Galactosylceramide (α-GalCer) is the prototypic agonist, but its excessive potency with simultaneous production of both pro- and anti-inflammatory cytokines hampers its potential therapeutic use. In search for novel agonists, we have analyzed the structure and function of HS44, a synthetic aminocyclitolic ceramide analog designed to avoid unrestrained iNKT cell activation. HS44 is a weaker agonist compared with α-GalCer in vitro, although in vivo it induces robust IFN-γ production, and highly reduced but still functional Th2 response. The characteristic cytokine storm produced upon α-GalCer activation was not induced. Consequently, HS44 induced a very efficient iNKT cell-dependent antitumoral response in B16 animal model. In addition, intranasal administration showed the capacity to induce lung inflammation and airway hyperreactivity, a cardinal asthma feature. Thus, HS44 is able to elicit functional Th1 or Th2 responses. Structural studies show that HS44 binds to CD1d with the same conformation as α-GalCer. The TCR binds to HS44 similarly as α-GalCer, but forms less contacts, thus explaining its weaker TCR affinity and, consequently, its weaker recognition by iNKT cells. The ability of this compound to activate an efficient, but not massive, tailored functional immune response makes it an attractive reagent for immune manipulation.

model, iNKT cell activation can result in very different outcomes: for example, they can induce asthma-like conditions, either alone or in combination with allergen challenge (13), or prevent this same pathological condition by stimulating a counteracting Th1 response (14, 15). Thus, their activation by glycolipid agonists has shown to be beneficial or detrimental, depending on the disease model and the type of immune response induced upon the particular treatment (11, 16).

Based on these capabilities, α-GalCer has been proposed as an immunomodulatory reagent with therapeutic capabilities (17, 18). Clinical trials using α-GalCer in several tumor conditions have been performed showing its safety, but results are far from the expectations raised by animal model studies. Two main factors are held responsible for the relative failure in its therapeutic application, as follows: the massive induction of a cytokine storm with concurrent and contradictory or even antagonistic functionalities and the profound anergic state induced on iNKT after their massive activation (19).

In an effort to selectively elicit either a Th1- or Th2-biased response upon Ag challenge, structural analogs of α-GalCer have been developed. Modifications include either the lipid tails responsible for CD1d binding or the sugar moiety that directly interacts with the TCR. In general, ligands that increase stability of the CD1d-ligand complex, such as those including aromatic rings in their fatty acid tail at certain positions, induce a Th1-biased immune response (20), whereas analogs that decrease the stability of the complex, such as OCH, which contains a truncated sphingosine chain, or C20:2 with a diunsaturated acid chain, tend to induce a Th2 bias (21, 22). Nevertheless, effects on iNKT activation are difficult to predict, as at least part of their functional behavior depends on the route of ligand presentation, the cellular compartments where processing and loading to CD1d (which also differs between human and mouse) (23) take place, and their efficient inclusion on lipid rafts, as well as on the presenting APC (24–26). In addition, their differential recognition by TCR depends on indirect conformational changes induced in CD1d structure (27).

Structural analogs that alter the polar head that is directly recognized by the TCR have also been synthesized. These ligands do not alter intracellular processing, as they share the lipid chain with α-GalCer or previously synthesized analogs; thus, their influence on iNKT activation would only derive from the stability of the binding to CD1d and the direct interaction with the semi-invariant TCR. The three-dimensional structure of the CD1d/α-GalCer/TCR complex (28), as well as other ligands, has clearly established a new rigid binding mode compared with MHC recognition, based on a lock-and-key mechanism that mediates TCR interaction (3). The interaction is dominated by the invariant Vα chain that recognizes both CD1d residues and the sugar head of the ligand, whereas Vβ exclusively interacts with CD1d α helices. Thus, the nature of the polar moiety and its molecular details are directly sensed by the dominating Vα chain and determine the affinity of the interaction, modulated by the Vβ chain (29, 30).

Alterations of specific positions in the sugar ring determine the affinity of the TCR interaction and, therefore, the efficiency and the tone of the iNKT activation. Nevertheless, the structure–function relationship in the recognition event is still not well understood. Agonists resistant to enzymatic degradations, such as α-galactosylceramide with a carbon-based glycosidic linkage, are extremely potent inducers of the Th1 response in vivo, even if they are poorly recognized in direct recognition assays, due to the low TCR affinity (31, 32). Prolonged biosubponibibility and long-lasting iNKT/APC interaction seem to be more effective in secondary transactivation of NK cells, the major source of IFN-γ in the iNKT-induced response, and in inducing a stronger Th1 response, with superior therapeutic effects in mouse models of cancer and microbial infections (31, 33). To add complexity to the search of therapeutic reagents, the human iNKT cells do not respond equally as mouse iNKT to glycolipid agonists, as evidenced by the weak response to OCH and α-C-GalCer (24).

Given all these considerations, the search for efficient iNKT agonists with functional differences compared with α-GalCer is an ongoing goal in the field, which attracts the work of many laboratories. In this study, we show the functional and structural properties of a new analog designed to have a weaker affinity for the TCR, while having a prolonged biosubponibibility to induce an efficient immune response. Based on a nonglycolipid architecture, in which the sugar moiety is substituted by a carba cyclitol ring and the O glycosidic linkage to the ceramide exchanged by an amino linkage resistant to glycosidase degradation, the amino cyclitol ceramide HS44 mimics the glucose configuration of the glycolipidic sugar. We have compared the functional activation of iNKT cells induced by HS44 with the one induced by α-GalCer, analyzing proliferation, cytokine production, and efficiency of immune response in a tumor and asthma model. In addition, biophysical measurements and three-dimensional structural characterization have been performed to define the interaction of the CD1d/HS44 complex with the semi-invariant TCR.

Materials and Methods

Mice

C57BL/6 female mice (6–8 wk old) were either purchased from Crlifa and used at the Animal Facility of the Universitat Autònoma de Barcelona, under protocols approved by the Ethnic Committee on Animal and Human Experimentation, or bred in the Animal Facility of the Instituto de Salud Carlos III (Madrid, Spain) and used under protocols approved by the Ethnic Committee on Animal Experimentation and Wellness. Female BALB/c Byj mice (6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free mouse colony at the Keck School of Medicine, University of Southern California, under protocols approved by the Institutional Animal Care and Use Committee.

Spleen cell cultures

Splenocytes from C57BL/6 mice were obtained by smashing the spleen and removing RBC’s with lysing buffer (Sigma-Aldrich). A total of 5 × 10⁴ cells was incubated with indicated amounts of KRN7000 (α-GalCer) (a gift of G. González-Aseguínolaza, Universidad de Navarra), OCH (A. Stout, National Institutes of Health Tetramer Facility), or HS44 arabinogalactan-ceramide in RPMI 1640 supplemented with 10% FCS, 50 μM 2-ME, 2 mM L-glutamine, and 1% methanol, in 96-well plates. For IL-4 and IFN-γ determinations, cell-free supernatants of day 4 were collected, and cytokine levels were quantified by ELISA (eBioscience) following manufacturer’s instructions. At least three different experiments were performed. Statistical significance observed between the different analogs was analyzed using the Student t test, and differences were considered significant at *p < 0.05 and **p < 0.01.

Amino cyclitol compounds were resuspended in 100% methanol or 100% DMSO at 1 mg/ml, and a use stock at 100 μg/ml was prepared. Compounds were heated at 56°C for 10 min and sonicated, before being diluted in complete culture media to a final concentration of 1% vehicle in the stimulation cellular assay.

Flow cytometry analysis

Briefly, spleen cultures stimulated with HS44 or OCH at 1 μg/ml or 100 ng/ml (α-GalCer) were preincubated with anti-CD16 (clone 2G42). After washing, cells were resuspended in staining media with FITC-conjugated anti-mouse TCRβ (clone H57-597; BD Pharmingen) and PE-conjugated anti-mouse CD11c (clone N418), anti-mouse CD62L (clone eBio35-17.2), CD11c (clone N418), CD62L (clone 492, and anti-mouse CD8 (clone eBio27.12). Cells were washed and fixed in 1% paraformaldehyde before being analyzed using CellQuest software (BD Biosciences).

NKT cell enrichment and in vitro culture

iNKT cell lines were prepared, as described before (34), with some modifications. Briefly, iNKT cells were negatively selected from splenocytes of wild-type BALB/c mice, using a mixture of PE-conjugated mAbs against CD8 (clone eBio35-17.2), CD11c (clone N418), CD62L (clone 492, and anti-mouse CD8 (clone eBio27.12). Cells were washed and fixed in 1% paraformaldehyde before being analyzed using CellQuest software (BD Biosciences).
MEL-14), and CD19 (clone eBioscience). All Abs were purchased from eBioscience. The samples were then stained with anti-PE microbeads mAb (Miltenyi Biotec) prior to being enriched using magnetic cell sorting. The percentage of iNKT cells was assessed by flow cytometry (15–20%) using a FACSCanto II eight-color flow cytometry (BD Biosciences), gated on the CD3⁺ α-GC/CD1d tetramer⁺ αβTCR⁺ cells. According to the percentage of purification, negatively enriched iNKT cells were then put in culture with RPMI 1640 supplemented with 10% calf serum (equivalent to 2 × 10⁷ NKT cells/well) in presence or absence of increasing concentration of α-GalCer, HS44, or OCH.

Quantitative real-time PCR

Total RNA was extracted from in vitro culture cells using the RNeasy mini kit (Qiagen), and cDNAs were generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s recommendations. Quantification of mRNA levels was carried out by quantitative real-time PCR on a CFX96 thermal cycler (Bio-Rad) with pre-designed TaqMan gene expression assays for actinβ (Mm00469685_m1), IL-4 (Mm00445259_m1), IFN-γ (Mm00400609_m1), IL-2 (Mm99999222_m1), IL-10 (Mm00439616_m1), IL-5 (Mm9999963_m1), and IL-17A (Mm00439619_m1) (Applied Biosystems), according to the manufacturer’s instructions.

Determination of in vivo cytokines

Aminocyclitol analog and α-GalCer in 200 μl PBS, 2% DMSO were injected i.p. to C57BL/6 mice. Sera were collected at 2 h and 21 h, and cytokines were quantified by cytometric bead assay (Th1-Th2-Th17 cytometric bead assay [CBA] kit; BD Biosciences), according to manufacturer’s instructions.

Induction of airway hyperreactivity and measurement of airway responsiveness

For measurement of airway hyperreactivity (AHR), mice were immunized intranasally with 1 μg α-GalCer or HS44 glycolipids in 50 μl PBS. AHR responses were assessed 24 h later by methacholine-induced airflow obstruction in conscious mice placed in a whole body plethysmograph (Buxco Electronics), as described before (13). In some experiments, we assessed AHR by invasive measurement of airway resistance, in which anesthetized and tracheostomized mice were mechanically ventilated using a modified version of a described method (34). Aerosolized methacholine was administered in increasing concentrations of methacholine, and we continuously computed airway resistance (Rₐ) and dynamic compliance (Cdyn) by fitting flow, volume, and pressure to an equation of motion.

Collection of bronchoalveolar lavage fluid and lung histology

After the measurement of AHR, a lethal dose of phenobarbital (450 mg per kg body weight) was administered i.p. to mice, the trachea was cannulated, the lung was then lavaged twice with 1 ml PBS supplemented with 2% FCS, and the fluid was pooled, as previously described (13, 34). After the bronchoalveolar lavage (BAL) was performed, lungs were removed, washed with PBS, fixed in 10% formalin, and stained with periodic acid Schiff (PAS) and H&E. The relative number of different types of leukocytes (lung cell differential) was determined from slide preparations of BAL fluid stained with H&E.

Determination of B16 melanoma lung metastases

C57BL/6 mice were i.v. challenged with syngeneic B16F10 melanoma, resuspended in RPMI 1640. A total of 500,000 cells in 200 μl was administrated 3 d after administration of indicated doses of iNKT agonists. Two weeks after challenge, mice were killed, lungs removed, and the number of metastatic nodules was visually counted.

Protein production

The expression and purification of fully glycosylated mouse CD1d/β₂-microglobulin (β₂m) heterodimer were carried out using the baculovirus expression system, as reported previously (35, 36).

Vα14-Vβ8.2 TCR refolding

TCRs and TCRβ chains were expressed in Escherichia coli inclusion bodies and refolded together by step-wise dialysis, according to established methods (35). After final dialysis, refolded TCR was bound to DEAE Sepharose beads and eluted with 100 mM NaCl in 10 mM Tris-HCl (pH 8), and further purified to homogeneity by anion exchange and size exclusion chromatography, as reported previously (35).

Glycolipid loading and ternary complex formation

Mouse CD1d was loaded overnight with 3–6 molar excess of HS44 (1 mg/ml in DMSO) in presence of 0.05% Tween 20 and 100 mM Tris-Cl (pH 7.0). HS44-loaded CD1d was purified by size exclusion chromatography first, and then incubated with equimolar amount of TCR for 30 min. Finally, the formed mCD1d-HS44-TCR ternary complex was purified by a second size exclusion chromatography. The complex was concentrated to 3.4 mg/ml in 10 mM HEPES (pH 7.5), 30 mM NaCl for subsequent crystallization.

Surface plasmon resonance studies

Surface plasmon resonance studies were performed using a Biacore 3000 (Biacore), according to the methods described previously (37). Biotin-labeled mCD1d was loaded with α-GalCer and HS44 (1 mg/ml in DMSO) overnight, and 200 resonance units mCD1d-glycolipid complex was immobilized on a streptavidin sensor chip (Biacore). The TCR was diluted in running buffer (10 mM HEPES, 150 mM NaCl, and 3 mM EDTA [pH 7.4]), and a series of increasing concentrations (0.02–1.25 μM) of the TCR in duplicate were passed over the mCD1d–glycolipid complex at 25°C with a flow rate of 30 μl/min. The experiments were repeated twice with a different TCR preparation. Kinetic parameters were calculated by subtracting the response of TCR to empty mCD1d molecules using a single Langmuir 1:1 model in the BIA evaluation software version 4.1.

Crystallization and structure determination

Crystals of mCD1d–HS44–TCR complexes were grown at 22.3°C by sitting drop vapor diffusion while mixing 0.5 μl protein with 0.5 μl precipitant (16% polyethylene glycol 3350, 8% v/v Tacsimate [pH 5.0]). Crystals were flash cooled at 100 K in mother liquor containing 20% glycerol. Diffraction data were collected at the Stanford Synchrotron Radiation Lightsource beamline 9.2 and processed with the mosflm software (38). The mCD1d-HS44-TCR crystal belongs to space group C2221, with cell parameters a = 78.78 Å; b = 191.66 Å; c = 150.92 Å. The asymmetric unit contains one mCD1d-glycolipid-TCR molecule with an estimated solvent content of 57.68%. The crystal structure (see Fig. 6) was determined by molecular replacement using MOLREX as part of the CCP4 suite (39, 40) using the protein coordinates from the mCD1d-G3B3 structure (Protein Data Bank code 2QTY) (41), followed by the Vα14Vβ8.2 TCR (from Protein Data Bank code 3QUZ) as the search model. After the molecular replacement solution was obtained containing both mCD1d and TCR, the model was rebuilt into σa-weighted 2Fₑ–Fₑ and Fₑ–Fₑ difference electron density maps using the program COOT (42). The final refinement steps were performed using the TLS procedure in REFMAC (43) with five anisotropic domains (α/α-ζ domain of CD1d, including carbohydrates and lipid, α-ζ-domain, β₂m, variable domain, and constant domain of TCR). The mCD1d-HS4-TCR structure was refined to 2.8 Å to a Rcryst and Rfree of 22.7 and 26.8%, respectively. The quality of the model was examined with the program Molprobity (44). Data collection and refinement statistics are presented in Table I. Coordinates and structure factors have been submitted to the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) under accession number 3RTQ.

Results

In vitro activation of iNKT cells by HS44

We have previously shown that NKT cells are activated in in vitro cell cultures by a novel series of α-GalCer synthetic analogs in which the sugar ring is substituted by a cyclitol with different sequences of its recognition. First, we checked its capacity to induce iNKT cell proliferation in an in vitro culture system, as a measurement of its capacity to specifically activate this immune cell population. We cultured single spleen cell suspensions in the presence of HS44 and compared the expansion of iNKT cells induced upon HS44 recognition with Th1 and Th2 prototypical iNKT agonists α-GalCer and OCH.
respectively. As shown in Fig. 1, HS44 induces expansion of CD1d-α-GalCer tetramer-labeled lymphocytes, specifically defining type I iNKT cells, in a weaker manner than either α-GalCer or OCH. In the case of α-GalCer, we used a 100 ng/ml concentration at which maximum stimulation is achieved. Analogs that are not recognized by iNKT cells (such as HS58, which is identical to HS44 except for a β orientation of the N-linkage between polar aminocylitol head and ceramide) do not induce any expansion of iNKTs.

**HS44 induces iNKT cell cytokine expression**

The ability to produce both Th1 and Th2 cytokines is a hallmark of iNKT cell activation. To analyze the iNKT cell response toward HS44, we measured the production of IFN-γ and IL-4 in an in vitro spleen cell culture assay in which splenocytes were cultured in the presence or absence of increasing amounts of HS44 and compared with the production in response to α-GalCer and OCH. As shown in Fig. 2A, HS44 induces secretion of both IFN-γ and IL-4, although in a weaker fashion than α-GalCer or the Th2 inducer OCH, coincidental with previous report (45). The induction of IFN-γ at the plateau level was far from the level induced by either α-GalCer or OCH (8–10 times lower), and the response was saturated at higher concentrations (~300 ng/ml versus 33 ng/ml concentration for α-GalCer). In the case of IL-4, the difference was not so dramatic, accounting for a 3-fold difference. Thus, the ratio of IFN-γ/IL-4 production is lower than when activating with α-GalCer and drops with decreasing HS44 concentrations (from 2.5 to 1) as occurs with OCH stimulation (IFN-γ/IL-4 ratio drops from 10 to 2.5 at 10 ng/ml), as opposed to the stable ratio of ~8 for α-GalCer, suggesting a certain Th2 bias in the HS44 activation of iNKT cells.

We further assessed the mRNA cytokine profile of HS44-treated iNKT cells by real-time PCR and compared the results with those obtained from NKT cells activated with the two glycolipids, α-GalCer and OCH. To avoid activation of iNKT cells, splenocytes from BALB/c mice were negatively enriched for iNKT cell, as described in Materials and Methods. The iNKT-enriched fraction was then cultured in presence or absence of increasing concentrations of HS44, α-GalCer, or OCH. The expression of IL-4, IFN-γ, IL-2, IL-10, IL-5, and IL-17A cytokines was then assessed by real-time PCR after 12 or 24 h of in vitro stimulation (Fig. 2B).

As expected, in the presence of α-GalCer or OCH iNKT, cells expressed significantly higher levels of IL-4, IFN-γ, IL-2, IL-10, and IL-17A mRNA after 12 h of stimulation. After 24 h of stimulation, IL-4 and IL-2 RNA expression levels were even significantly higher (up to 6-fold increased), and also IL-5 was induced. In the presence of HS44, iNKT cells also significantly increased cytokine RNA expression, although expressed IL-4, IFN-γ, and IL-2 RNA levels were lower as compared with α-GalCer- and OCH-treated cells (~3-fold difference at the highest concentration tested after 12 h of stimulation), thus correlating with protein quantification. Contrary to α-GalCer and OCH, HS44-activated iNKT cells did not show increased IL-2 induction at 24 h, and IL-4 mRNA expression returned to constitutive levels. Induction of IL-4 mRNA at 12 h was much higher than IFN-γ (~5-fold difference) and was sustained at lower HS44 concentrations (up to 30 ng/ml versus 250 ng/ml for IFN-γ), again suggesting a certain Th2 bias at initial times. Moreover, the level of IFN-γ and IL-4 expression was significantly decreased after 24 h of culture, suggesting that activation by HS44 is not long lasting in comparison with the other two glycolipids. In contrast, cytokines that were less relevantly induced by prototypic agonists (IL-5, IL-10, and IL-17) are similarly induced by HS44, indicating a certain basal level of cytokine induction after iNKT activation independently of agonist strength.

**HS44 selectively induces Th1 cytokine production in vivo**

To study the physiological activity of HS44 in the activation of iNKT cells and its immediate downstream effects, we i.p. ad-

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**FIGURE 1.** Expansion of iNKT cells upon treatment with HS44. Spleen cells from B6 mice were cultured in the presence or absence of 1 μg/ml HS44 or OCH or 100 ng/ml α-GalCer for 4 d. Flow cytometry analysis of splenocytes labeled with CD1d-PBS57-PE tetramer and anti-TCR FITC. iNKT percentages (double-positive cells) were calculated among electronically gated lymphocyte population. A representative of two different experiments is shown.
administered different amounts of HS44 and α-GalCer to B6 mice and analyzed the serum cytokine levels at two different time points, 2 h and 21 h, coincident with the times in which maximum levels of IL-4 and IFN-γ are reached. By means of a CBA, we measured the levels of IFN-γ, TNF, IL-4, IL-2, IL-5, IL-10, and IL-17. In vivo HS44 administration induced a potent production of IFN-γ at 21 h at high dose (1 μg), similar to the amount induced by α-GalCer at the same dose (<2-fold difference), in clear contrast to the comparatively weak production found in in vitro stimulation assays (Fig. 3). HS44 differential activity was mainly found in the dose-response profile, because whereas α-GalCer maintained the maximum stimulatory capacity at 200 ng, HS44 dropped to levels equivalent to an α-GalCer dose of 40 ng, which accounts for a 5-fold higher potency than the analog. The other Th1 cytokine measured in the assay, TNF, was very poorly induced, although production at 21 h was consistently higher than at 2-h time, whereas α-GalCer induced a strong response at 2 h that was not sustained over time.

On the contrary, IL-4 was only poorly induced and only at high doses in vivo, very differently from α-GalCer (or OCH; C.M. Barra and A.R. Castaño, unpublished observations), which correlates more with the poor mRNA induction at longer times in vitro (Fig. 2B). ELISA analysis of IFN-γ and IL-4 levels corroborated these results, although a general underestimation of ∼50% of serum IL-4 by CBA could be noted. In these ELISA quantifications, IL-4 levels induced by HS44 at 1 μg dose were higher and IL-4 could also be detected at 200 ng dose of HS44 (C.M. Barra and A. R. Castaño, unpublished observations), not altering above conclusion. Similarly, IL-2 and IL-6 levels in serum were also poorly increased, in contrast to the high amounts found in animals treated with a high dose (1 μg) of α-GalCer. IL-10 and IL-17, on the contrary, were very poorly induced by either α-GalCer or HS44, in concordance with the weak mRNA induction in in vitro experiments (the amounts detected were too close to background as to allow reliable comparisons). Thus, in sharp contrast to in vitro stimulation, HS44 induces a strong production of IFN-γ in treated animal, with similar efficiency as α-GalCer, although with lower potency, whereas systemic induction of IL-4 was much lower than expected, as were other cytokines typically produced in response to α-GalCer treatment. The cytokine storm characteristic of the overt superactivation induced by α-GalCer was thus restricted to specific high IFN-γ production.

Antitumoral activity induced by activation of iNKT with HS44

Because HS44 induced a stronger than anticipated in vivo production of prototypic Th1 cytokine IFN-γ, we reasoned that it might be effective in disease models that require a Th1-type immune response for their control. The metastases in the lung of the B16F10 melanoma tumor cell line is one of such models in which iNKT activation prevents the establishment of lung metastases after challenge with the tumor, through the sequential production of IFN-γ by iNKT and NK cells (46).

To investigate the potential role of HS44 to induce an effective antitumoral Th1 response, we i.v. administered different doses of either HS44 or α-GalCer to B6 mice, 3 d before challenge with syngeneic B16F10 melanoma cells. Two weeks later, mice were sacrificed, lungs were extracted, and lung metastatic nodules were quantified. As expected, α-GalCer administration prevented establishment of metastases up to a 10 ng dose per animal (Fig. 4). HS44 was also highly efficient in preventing establishment of metastasis at 200 ng and 100 ng per mice dose, and even at 10 ng still showed a relevant control of the numbers of metastasis (<50% of maximum quantifiable). Thus, HS44 was only slightly less potent than α-GalCer, as the small differences found in antitumoral effect when comparing same doses of both treatments were not statistically significant. Of note, we used a more stringent assay than previously published, administering iNKT agonists 3 d earlier than tumor cells and challenging with 500,000 B16 cells instead of the 2-d difference and 200,000 cells used in other studies (33). Thus, despite the low level of iNKT activation
in vitro, HS44 was able to mount a very strong Th1 response exemplified by efficient tumor suppression that effectively prevented the establishment of metastases. The antitumoral response activated by HS44 not only parallels the high induction of IFN-γ, but in fact is significantly more potent, as it takes place at doses (10 ng) in which systemic Th1 cytokine production is minimal.

Activation of pulmonary iNKT cells with HS44 induces AHR

Despite the low induction of systemic IL-4 in B6 mice, the in vitro experiments both in B6 and BALB/c mice indicated that HS44-mediated activation of iNKT cells was able to induce both RNA cytokine expression and secretion, at least at early time points. Therefore, we resorted to determine the capacity of HS44 to induce a physiologically relevant Th2 response in a mouse strain prone to produce Th2 immune responses and in a characteristic disease model. We analyzed the specific role of HS44 in the development of AHR, and compared it with glycolipids such as α-GalCer, by administering intranasally to naïve BALB/c mice the iNKT agonists. BALB/c mice challenged with HS44 or α-GalCer developed severe AHR by 24 h measured as enhanced pause (Fig. 5A), although the AHR level obtained from HS44-treated group was significantly lower when compared with α-GalCer-treated group.

AHR was also confirmed by direct measurement of RL and Cdyn (a measure of the elasticity of the lung) in anesthetized, tracheotomized, intubated, and mechanically ventilated wild-type BALB/c (Fig. 5B). Although RL showed a lower induction of hyperreactivity, lung elasticity was equally reduced by HS44 and α-GalCer. The difference in the level of AHR between HS44- and α-GalCer–sensitized mice was also correlated with the total cell infiltrate obtained from BAL (Fig. 5C). We examined the lung histology of mice from Fig. 5A by H&E to determine cellular infiltration and by PAS staining to determine mucus production. As expected, lung tissues of α-GalCer–sensitized mice showed extensive cellular infiltration surrounding the airways and

FIGURE 3. Serum cytokine production after HS44 administration. Indicated amounts of HS44 or α-GalCer were i.p. administered to B6 mice. Blood samples were collected 2 h and 21 h later and cytokine levels were determined by CBA. Data indicate the mean of three different mice. A representative of three different experiments is shown.

FIGURE 4. HS44 antitumoral activity controls establishment of melanoma metastases. C57BL/6 mice were i.v. treated with indicated amounts of either HS44 or α-GalCer or with vehicle 3 d before i.v. challenge with 5 × 10⁶ B16 melanoma cells. Two weeks later, the lungs were extracted for metastases quantification. A representative experiment of two is shown, with five mice per condition. Horizontal continuous lines show statistical differences between relevant treatments, with *p < 0.05, **p < 0.01. Discontinuous lines indicate relevant treatments without statistically significant differences. Photographic images from a representative mouse and treatment are shown.
thickened membrane and mucus production (Fig. 5D). The lung histology of HS44-sensitized mice showed a lower airway inflammation in comparison with what was observed in the α-GalCer–sensitized mice, but still a very significant cellular infiltration, with a similar mucus production. Overall, these results suggest that intranasal administration of HS44 in mice induces moderate AHR characterized by moderate airway inflammation and mucus production.

Biophysical characterization of HS44 recognition by semi-invariant TCR

To analyze the structural basis that may help explain the presented functional data, we analyzed the biophysical interaction between the HS44–CD1d complex and a TCR representative of iNKT cells [derived from iNKT clone 2C12 (47)]. We measured the equilibrium-binding constants of the refolded Vα14Vβ8.2 TCR toward α-GalCer and HS44 bound to mouse CD1d by surface plasmon resonance (Fig. 6A). With an equilibrium-binding constant (K_D) of 155 nM, the TCR-binding affinity toward HS44 is considerably weaker than that of α-GalCer [11.2 nM (37)]. The kinetic parameters reveal that the TCR binds HS44 and α-GalCer with a similar association rate (K_a = 1.26 × 10^5 M^−1 s^−1 and 1.30 × 10^5 M^−1 s^−1), respectively). However, the dissociation of HS44 (K_d = 19.5 × 10^3 s^−1) is 10–15 times faster than α-GalCer (K_d = 1.45 × 10^3 s^−1). In summary, the kinetic data indicate that the initial binding of the TCR to mCD1d-HS44 and mCD1d–α-GalCer complexes is very similar. This suggests that HS44 and α-GalCer bind similarly to CD1d before TCR engagement. As a result, the chemical differences between HS44 and α-GalCer are mostly affecting the dissociation rate, and thus the stability of the CD1d–glycolipid–TCR complexes, which translates in a less potent activation of iNKT, compared with α-GalCer.

CD1d-HS44-TCR structure

To reveal the structural basis for HS44 Ag recognition by mouse iNKT TCRs and to understand how the replacement of the galactose of α-GalCer with an aminocyclitol group still retains relatively high TCR-binding affinity, we have determined the crystal structure of the ternary complex with the HS44 Ag, at a resolution of 2.8 Å (Fig. 6B, Table I). The crystal structure exhibits very clear electron density for the glycolipid ligand, suggesting an ordered orientation upon TCR engagement (Fig. 6C). As expected, HS44 binding to CD1d, as well as TCR binding to CD1d-HS44, is overall very similar to α-GalCer (48). HS44 is bound to mCD1d with its phytosphingosine chain inserted in the Fp pocket and the long chain fatty acid in the Ap pocket. The aminocyclitol head is exposed similarly at the CD1d-binding groove for TCR recognition (Fig. 6). Both HS44 and α-GalCer bind very similar to mCD1d in the ternary complex (Figs. 6D, 7). H bonds between both ligands and CD1d residues are conserved, indicating that the chemical differences between the polar moieties of HS44 and α-GalCer (N versus O-glycosidic linkage, aminocyclitol versus galactose, respectively) do not alter the lipid binding to CD1d, but rather affect TCR interaction. Significantly, the aminocyclitol defining N that links the cyclitol with the ceramide in substitution of glycosidic O, retains the H bond with T156.

thickened membrane and mucus production (Fig. 5D). The lung histology of HS44-sensitized mice showed a lower airway inflammation in comparison with what was observed in the α-GalCer–sensitized mice, but still a very significant cellular infiltration, with a similar mucus production. Overall, these results suggest that intranasal administration of HS44 in mice induces moderate AHR characterized by moderate airway inflammation and mucus production.

Biophysical characterization of HS44 recognition by semi-invariant TCR

To analyze the structural basis that may help explain the presented functional data, we analyzed the biophysical interaction between the HS44–CD1d complex and a TCR representative of iNKT cells [derived from iNKT clone 2C12 (47)]. We measured the equilibrium-binding constants of the refolded Vα14Vβ8.2 TCR toward α-GalCer and HS44 bound to mouse CD1d by surface plasmon resonance (Fig. 6A). With an equilibrium-binding constant (K_D) of 155 nM, the TCR-binding affinity toward HS44 is considerably weaker than that of α-GalCer [11.2 nM (37)]. The kinetic parameters reveal that the TCR binds HS44 and α-GalCer with a similar association rate (K_a = 1.26 × 10^5 M^−1 s^−1 and 1.30 × 10^5 M^−1 s^−1). However, the dissociation of HS44 (K_d = 19.5 × 10^3 s^−1) is 10–15 times faster than α-GalCer (K_d = 1.45 × 10^3 s^−1). In summary, the kinetic data indicate that the initial binding of the TCR to mCD1d-HS44 and mCD1d–α-GalCer complexes is very similar. This suggests that HS44 and α-GalCer bind similarly to CD1d before TCR engagement. As a result, the chemical differences between HS44 and α-GalCer are mostly affecting the dissociation rate, and thus the stability of the CD1d–glycolipid–TCR complexes, which translates in a less potent activation of iNKT, compared with α-GalCer.

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FIGURE 6. TCR-binding kinetics and HS44 ternary complex crystal structure and stereo view of HS44 electron density and HS44 binding to mCD1d. (A) A representative sensorgram of the TCR binding to mCD1d–HS44 complex is shown (from three independent experiments). Each colored curve depicts a different concentration of injected TCR. (B) Crystal structure of the mCD1d–HS44–TCR ternary complex is shown. HS44 is shown as yellow sticks, between mCD1d (gray) and TCR (α-chain in cyan; TCR β-chain in orange). Chemical structures of HS44 and α-GalCer for comparison are depicted with the galactose and aminocyclitol head groups highlighted in red. (C) Representation of the final 2Fo-Fc map drawn around glycolipid HS44 from the ternary mCD1d–HS44–TCR complex. The 2FoFc electron density map is contoured for HS44 at 1σ and drawn as a blue mesh in a side view. Hydrophobic mCD1d residues interacting with the lipid backbone and charged residues contacting the polar moieties of the glycolipid aminocyclitol are depicted. (D) HS44 and α-GalCer are shown superimposed in the mCD1d-binding groove. The aminocyclitol head of HS44 (yellow) and galactose head of α-GalCer (green) are exposed similarly for TCR recognition. Binding to CD1d is very similar for both ligands. H bonds between mCD1d and HS44 are highlighted in blue dash lines and involve the CD1d residues D80, D153, and T156. α-GalCer is shown in green; HS44 is shown in yellow; mCD1d H chain and β,m are shown in gray. Oxygen is depicted in red, and nitrogen in blue.

The iNKT cell TCR docks parallel to the CD1d-binding cleft, with the α-chain above the F pocket (Figs. 6, 7). The TCR α-chain exclusively contacts the aminocyclitol ligand through its CDR1 and CDR3 regions, whereas TCR β- and γ-chains together form conserved contacts with CD1d residues, mainly through CDR3α, CDR2β, and CDR3β (Fig. 7).

TCR–lipid interaction

To compare TCR recognition of HS44 with α-GalCer, the interactions between TCR and both lipids were analyzed (Fig. 7, Table II). In the α-GalCer complex, the 2′ hydroxyl of the galactose ring head forms an H bond with Gly96β of CDR3α, whereas the 3′ and 4′ hydroxyl forms H bond to Asn30α of CDR1α. However, note that the H bond contact between the 4′OH of HS44 and N30α of TCR is lost, due to the structural differences between the aminocyclitol ring, which is more similar to glucose (equatorial configuration of the HS44 hydroxyl versus the opposite axial orientation in α-GalCer), and the galactose. Instead, a water molecule mediates an H bond between the 3′OH and 4′OH of HS44 and N30α of TCR (Fig. 7, top right), whereas the rest of the contacts are retained. In total, α-GalCer forms four H bonds with the TCR, whereas HS44 forms only three direct H bonds with TCR, in addition to the water-mediated H bond, leading to a reduced interaction with the TCR. In conclusion, and in agreement with other α-GalCer analog structures (49), the loss of H bonds between the TCR and the Ag leads to a faster TCR dissociation and overall reduced binding affinity, thus explaining the generally weaker potency of HS44 compared with α-GalCer.

Discussion

iNKT cells are innate type of lymphocytes that recognize glycolipid ligands restricted by CD1d, inducing their immediate activation, which implies a rapid and massive production of cytokines and the downstream transactivation of dendritic cells (DC), T cells, and B cells. Due to this capacity to be activated in early phases of the immune response, iNKT are potent immunoregulatory cells that have been shown to participate in antimicrobial, antitumoral, and autoimmune responses (11, 12). Because of this capacity to control the immune response by their direct activation with pharmaceutical deliverable agonists, they have been proposed as immunotherapeutic targets of first interest. α-GalCer, the prototypical ligand recognized by all iNKT cells, has been the subject of an intense investigation in animal models of human diseases, showing its efficacy in the activation of efficient antimicrobial and antitumoral responses and in the amelioration or potentiation of autoimmune diseases, depending on the disease model and paucity of treatment (16, 18). It is a clinical trial phase I therapeutic, but its effects are far from the expectations derived from animal model studies.

Applicability of α-GalCer as an immunomodulatory reagent that may be useful as immunotherapeutic tool is hampered by two intrinsic characteristics, as follows: its extraordinary potency,
based on its capacity to activate all iNKT cells inducing their full activation characterized by a cytokine storm with counteracting Th1, Th2, and even Th17 activities, and the induction of a drastic and prolonged phase of anergy consequence of this excessive activation. The superantigen-like characteristics of α-GalCer recognition translate in the unpredictable consequences of its administration, in such a way that the activation pattern of the immune response depends on variables that are mechanistically poorly defined, such as dose, timing, repetitiveness, environment, via of administration, etc. (17). An empirical rather than rational approach, based on the experience of previous experiments, but not on the full understanding of the complex set of molecular mechanisms involved in activation of the immune system after α-GalCer recognition by iNKT cells, guides its use both in animal assays as well as in clinical trials. In addition, it is the target of catabolic degradation by glycosidases that limits its bioavailability, and presumably limits its efficacy in long-term treatment of chronic diseases, as cancer or autoimmunity. Because of these limitations, there is a great interest in obtaining CD1d ligands that modulate iNKT cell response in a more predictable and specific manner. Therefore, we resorted to search for new reagents that may overcome some of these problems and be of potential use in immunotherapy. We developed a series of analogs of the α-GalCer, based on the substitution of the sugar O-glycosidic–linked moiety of the glycolipid by a cyclitol head linked by an amino linkage to the ceramide, generally termed aminocyclitols, able to be recognized by NKT cells (45).

The aminocyclitol ligands that we have generated have the same lipid backbone as α-GalCer; therefore, their differential recognition and activation of iNKT will not be determined by differing CD1d-loading kinetics or distinct intracellular processing and presentation, factors that alter iNKT activation in a poorly predictable manner still under investigation (24, 27, 31). Instead, their modulatory effects will be solely determined by the polar head group, which varies between the different ligands, with the common influence on in vivo bioactivity derived from the resistance to glycosidase degradation imposed by their molecular architecture, that is, the cyclitol nature of the ring and its N linkage to the ceramide. The HS44 analog studied in this work has a cyclitol conformation that mimics the glucose ring, with the opposite 4’OH orientation relative to galactose sugar, except for the substitution of the C5 methanol group, which does not participate in either TCR recognition or CD1d binding, by an OH. As α-GlcCer is a less potent activator of iNKT cells, it was expected that HS44 would be a less potent agonist (8). In vitro studies showed that to be the case, with HS44 being able to be recognized by and activate iNKT cells in a weaker fashion than α-GalCer. Proliferation studies with whole spleen cell cultures and cytokine expression, both at protein and mRNA level, showed that both Th1- and Th2-biasing prototypical ligands, α-GalCer and OCH, were more potent activators. Particularly, secretion of both Th1- and Th2-defining cytokines, IFN-γ and IL-4, is drastically diminished upon activation with HS44, showing both less efficacy and less potency in in vitro experiments. Analysis of a larger panel of cytokines by mRNA profiling shows that α-GalCer, OCH, and HS44 have almost identical dose-dependent profiles for IL-10, IL-5, and IL-17 at 12 and 24 h postactivation. On the contrary, IFN-γ, IL-4, and IL-2 showed very different profiles depending on the agonist. Whereas α-GalCer and OCH show similar responses, except for the lower induction of IFN-γ by OCH at lower doses at 24 h (correlating with its known Th2-biasing response) (21), HS44 showed a drastic decrease of efficacy in inducing cytokine mRNA, especially at 24 h, suggesting that activation of iNKT is less potent and short lasting, comparing with full agonists.

One of the structural characteristics of HS44 that may have a major impact on its biological effects is the substitution of the O atom of the glycosidic linkage by an N (amino linkage), thus impeding the degradative action of intracellular and extracellular glycosidases, presumably allowing for a prolonged T1/T2 in vivo and a prolonged activation of iNKT cells. This has been suggested to be a major determinant of the reactivity of α-GalCer analogs that are poorly recognized in in vitro assays, due to a weak TCR recognition (31, 32), but that are potent agonists in vivo, inducing a potent Th1 response (33, 50). Administration of HS44 to model animals proved this to be the case. Opposite to what could be expected from in vitro experiments, HS44 induced an efficient IFN-γ response close in magnitude to α-GalCer, although with lower potency. This cytokine response was heavily biased, as none of the other cytokines induced by α-GalCer treatment that were tested was highly induced by HS44: only at high doses (1 μg per animal) were minor levels of TNF, IL-4, IL-2, and IL-6 found in the serum of treated animals. Consequently, with this highly specific Th1 cytokine induction, HS44 proved to be also highly efficient in inducing a potent antitumoral response. The B16 melanoma tumor model is a characteristic Th1 immune response model in which control of the establishment of metastases in the lung is totally dependent on the activation of a potent Th1 response characterized by a high production of IFN-γ, mainly by activation of NK cells secondary to iNKT activation (46). In this model, HS44 proved to be almost as efficient as α-GalCer in preventing the establishment of metastases with a very similar potency, despite being a much weaker in vitro agonist. In conclusion, the antitumor effect was far more efficient than systemic cytokine production, as metastatic growth was prevented at agonist doses in which serum IFN-γ levels were minimal. This indicates that either extremely high levels of IFN-γ are not
necessary for controlling metastasis implantation or that other secondary effector mechanisms, presumably more efficiently induced by HS44, also participate in a relevant manner in the antitumoral response.

It could be argued that inducing exclusively a Th1 response may not be as useful in the natural context of disease treatment as a more complete activation of the immune response, but biased to the specific effect required (51). In fact, efficient IFN-γ production by iNKT cells requires full IL-4 production capabilities at early stages of activation (52). We demonstrated that HS44 was able to induce Th2 immune responses in vivo, as intranasal administration of HS44 resulted in induction of moderate AHR, a complex inflammatory disorder caused by Th2-driven inflammatory responses (53–55). Several groups have previously shown that intranasal administration of glycolipids such as α-GalCer induces severe AHR by activating iNKT cells to produce significant levels of IL-4 and IL-13 (56). Although administration of HS44 resulted in a significantly lower airway function in comparison with the group treated with α-GalCer, the lung inflammation, mucus production, as well as lymphocyte infiltration in the lungs were comparable. All these biological assays show that HS44 is a iNKT agonist, able to mount efficient Th1 and Th2 immune responses with capacity to generate a very efficient antitumoral response and to induce autoimmune effects characteristic of allergic responses, in specific mice models.

The lower potency of HS44 in comparison with α-GalCer is the consequence of a lower interaction with the Vα14Jα18 TCR characteristic of iNKT. Surface plasma resonance experiments show that the KD of the TCR interaction with the HS44–CD1d complex is 155 nM, 14 times higher than for α-GalCer complex. This reduced affinity is exclusively due to a 13-fold faster dissociation kinetics of the TCR. This is in total agreement with recent measurements of another TCR interaction that showed that the affinity for α-GalCer is 10 times higher than for 49 deoxyα-GalCer and the affinity of OCH also is 10 times higher than for the equivalent glucose analog (that differs in the orientation of the sphinganine moiety).

The molecular interactions within the complex were analyzed using the program CONTACT (CCP4, 1994). Consistent with the analysis in Pellicci et al. (48), the cutoffs used were 4 Å (van der Waals interactions), 3.3 Å (hydrogen bonds), and 4.5 Å (salt bridges). Additional significant interactions with distances comparable with the cutoffs applied are also listed with the corresponding distance indicated in the Notes in the table.

### Table II. Molecular contacts in the mCD1d–HS44–iNKT TCR complex

<table>
<thead>
<tr>
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<th>mCD1d</th>
<th>Bonds</th>
<th>Notes</th>
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<td>H bond</td>
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<td>Salt bridge</td>
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<td>Asp94</td>
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<td>Arg79 NH1</td>
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<td>Ser68 O</td>
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<td>Gly96</td>
<td>C-2, 2’-O</td>
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4′OH as HS44 does), both of them due to a faster dissociation kinetics of the trimeric complex (49). In addition, a 6′ galactose-modified analog of α-GalCer, BnNH-GSL-1′, of which the head group is pulled toward the CD1 plane in the crystal structure, due to additional interactions with CD1d, also loses its 4′OH H-bond interaction with the TCR, resulting in an almost identical TCR-binding kinetics compared with HS44 (57). Therefore, we can conclude that the introduction of an Amino linkage of the polar cyclitol head to the ceramide and the substitution of the CH2-CH in the CS′ position by an OH have minor, if any, effects on the interaction with the TCR, and that the reduced affinity would mostly be the consequence of the altered conformation of the 4′OH. Also in concordance with above study, there is a strict correlation between TCR affinity and in vitro cytokine response. On the contrary, the proliferative response of activated iNKT cells is only partially affected by the diminished affinity, as previously published (32, 49), decreasing in a moderate manner compared with the large differences in interaction strength, indicating that proliferation does not directly correlate with affinity. Similarly, sustained in vivo production as well as secondary cell activation are only partially affected by lower affinity with the TCR and more dependent on the continuity of agonist presentation. Thus, the resistance to glycosidase degradation and, therefore, its prolonged bioavailability is a major factor for in vivo functionality more relevant than intrinsic affinity of the analog or the in vitro iNKT activation profile, especially in inducing antitumoral responses, which is more dependent on secondary activation of effector cells.

The structure of the trimeric complex explains these effects on affinity. The HS44–CD1d–TCR complex is highly similar to the α-GalCer complex, with differences affecting only at the contacts between the polar ring of HS44 with the CDR1α and CDR3α segments of the TCR, with minor rearrangements of few lateral chains involved in the interaction. HS44 adopts an almost identical orientation compared with α-GalCer, although slightly closer to the CD1d plane, maintaining the three hydrogen bonds at the entrance of binding groove that anchor the glycolipid to CD1d, including that of the glycosidic O that is maintained in the substituting polar N. In contrast, the recently solved structure of α-GlCer analogs shows a repositioning of the sugar along the A′ pocket and flattening against the CD1d plane (49). Thus, the TCR interaction with HS44 follows the rigid docking characteristic of α-GalCer–CD1d interaction, without the need for an induced fit of the ligand, indicated by the almost identical TCR association rate ($K_a$), compared with α-GalCer.

In summary, we have shown the functional and structural properties of an α-GalCer analog that may be useful in therapeutic applications. HS44 is characterized by a lower intrinsic capacity to activate iNKT cells, due to lower affinity with the semi-invariant TCR, which may be an advantageous property for its use as a modulatory agent (18, 58). The lower potency may not only be beneficial as to the induction of an efficient immune response, but also may signify a lower capacity to transactivate DC presenting self Ags and license cross-priming (59), thus reducing the possibility of stimulating an autoimmune response if used as either a therapeutic reagent or an adjuvant, a risk associated with the ability of stimulating an autoimmune response if used as either self Ags and license cross-priming (59), thus reducing the possibility of stimulating an autoimmune response if used as either

References
14. Matsuda, H., K. Takeda, T. Koya, M. Okamoto, Y. Shiraishi, N. Miyahara, Y. H., C.M.B., A.L., and A.R.C. are inventors on a pending patent application related to the use of aminocyclitols for disease treatments. The other authors have no financial conflicts of interest.


Corrections


The fourth author’s last name was published incorrectly. The correct name is Elisenda Alari-Pahissa.

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