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Improved Outcomes in NOD Mice Treated with a Novel Th2 Cytokine-Biasing NKT Cell Activator

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Activation of CD1d-restricted invariant NKT (iNKT) cells by α-galactosylceramide (αGalCer) significantly suppresses development of diabetes in NOD mice. The mechanisms of this protective effect are complex, involving both Th1 and Th2 cytokines and a network of regulatory cells including tolerogenic dendritic cells. In the current study, we evaluated a newly described synthetic αGalCer analog (C20:2) that elicits a Th2-biased cytokine response for its impact on disease progression and immunopathology in NOD mice. Treatment of NOD mice with αGalCer C20:2 significantly delayed and reduced the incidence of diabetes. This was associated with significant suppression of the late progression of insulitis, reduced infiltration of islets by autoreactive CD8+ T cells, and prevention of progressive disease-related changes in relative proportions of different subsets of dendritic cells in the draining pancreatic lymph nodes. Multiple favorable effects observed with αGalCer C20:2 were significantly more pronounced than those seen in direct comparisons with a closely related analog of αGalCer that stimulated a more mixed pattern of Th1 and Th2 cytokine secretion. Unlike a previously reported Th2-skewing murine iNKT cell agonist, the αGalCer C20:2 analog was strongly stimulatory for human iNKT cells and thus warrants further examination as a potential immunomodulatory agent for human disease.


Type 1 diabetes is an organ-specific autoimmune disease mediated by pathogenic Th1-type T cells that specifically destroy the pancreatic islet β cells. The NOD mouse is susceptible to the spontaneous development of type 1 diabetes and many studies support its usefulness as a model for this disease and its prevention or treatment in humans (1). Type 1 or invariant NKT (iNKT)4 cells are unconventional regulatory and effector T cells that recognize glycolipid Ags presented by CD1d (2). Upon activation, iNKT cells release copious amounts of both Th1 and Th2 cytokines such as IFN-γ and IL-4 and significantly influence the outcome of developing or ongoing adaptive immune responses. Multiple studies show that iNKT cells play a regulatory role in autoimmune diabetes in NOD mice (reviewed in Ref. 2), and treatment with synthetic α-galactosylceramides (αGalCer) that activate iNKT cells can block or attenuate the development of clinical diabetes in these animals (3–5). However, the mechanisms by which iNKT cell activation protects NOD mice from diabetes are still not well-understood and are likely to be multifactorial.

Previous work has shown that alterations of the basic lipid structure of the prototypical iNKT cell activator KRN7000 ([(2S, 3S, 4R)-1-O-(α-d-galactopyranosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol], in this study referred to as αGalCer C26:0) can lead to striking changes in the patterns of cytokine production elicited following iNKT cell activation (6–10). One of the most intensively studied examples of this is an αGalCer analog designated OCH, in which the fatty acid chain has been shortened to C24:0 and the sphingoid base truncated to C9. This analog is a relatively selective stimulator of IL-4 secretion and has pronounced anti-inflammatory effects in NOD mice and other mouse models of autoimmune diabetes (6, 11–15). Our earlier studies identified a novel derivative of KRN7000 containing an 11,14-cis-diunsaturated C20 fatty acid (αGalCer C20:2) as a potent iNKT cell activator that powerfully stimulates production of IL-4 in association with reduced production of IFN-γ. In this study, we have directly compared the iNKT cell-activating properties of the C20:2 and OCH compounds and find that the former is a substantially more potent iNKT cell agonist, particularly for human iNKT cells. In studies of diabetes prevention in NOD mice, we observed that C20:2 produced significant improvements in clinical and immunological outcomes compared with a closely related analog (αGalCer C24:0), which has similar potency but elicits a more

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4 Abbreviations used in this paper: iNKT, invariant NKT; αGalCer, α-galactosylceramide; PLN, pancreatic lymph node; DC, dendritic cell; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; Treg, regulatory T cell.
mixed cytokine response similar to KRN7000. These results point to important advantages of C20:2 for prevention of autoimmunity in NOD mice compared with other previously studied analogs of αGalCer and suggest that C20:2 warrants further investigation as a potential agent for prevention or treatment of human type 1 diabetes.

Materials and Methods

Mice
Female NOD/LtJ, C57BL/6, and BALB/c mice at 3–4 wk of age were purchased from The Jackson Laboratory. NOD/Mk mice at 3–4 wk of age were purchased from Taconic Farms. Mice were maintained in the animal facilities at Albert Einstein College of Medicine under specific pathogen-free conditions and were allowed to adapt to these housing conditions for 1–3 wk before initiating experimental protocols. The institutional animal welfare committee approved all experimental procedures.

Cell lines
Murine iNKT hybridomas D3N3A-1.2 (Vα14/Vβ8.2) and D3N3A-1.4 (Vα14/Vβ10), and murine CD1d-transfected A20 B lymphoma, were provided by Dr. M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA). Murine iNKT hybridoma N38.2H4 (Vα14/Vβ7) was provided by Dr. K. Hayakawa (Fox Chase Cancer Center, Philadelphia, PA). The human iNKT cell clone D2N.5 has been previously described (16) and other clones used for experiments referred to but not shown in the current manuscript were unpubished human iNKT cell clones derived in our laboratory from human peripheral blood (clones D2N.C4, D2N.C5, HDD.11, HDA.A7, and HDA.A8). H-2Kb-transfected murine RMA-S lymphoma cells (RMA-S/Kb cells) were provided by Dr. M. Bevan (University of Washington, Seattle, WA).

Glycolipid preparation
All glycolipids used in this study were synthesized according to previously reported methods (8, 17). For in vivo administration, glycolipids were dissolved in 200 μg/ml in PBS containing 0.5% Tween 20, heated to 80°C for 10 min, and sonicated for 5 min in a water bath sonicator (model 2510; Branson Ultrasonics) before use. The resulting suspensions were then diluted in prewarmed PBS to a glycolipid concentration of 16 μg/ml (0.04% final (Tweem 20)), sonicated, heated to 80°C, and immediately used for injection. For in vitro assays, glycolipids were dissolved in DMSO at a concentration of 500 μM, briefly sonicated, and then diluted directly into medium.

Diabetes treatment and monitoring
Starting at 4–6 wk of age, female NOD mice received seven weekly i.p. injections of glycolipid (4 μg/mouse in 250 μl of vehicle consisting of PBS plus 0.04% Tween 20) or vehicle. Diabetes was assessed starting at 10 wk of age by monitoring mice weekly for glucosuria with Diastix reagent strips (Bayer). Mice were considered diabetic when two consecutive positives were obtained and the time of onset of diabetes was scored 4.

Assessment of insulitis by histopathology
Pancreata were fixed in Bouin’s solution and sectioned at three nonoverlapping levels. Granulated β cells were stained with aldehyde fuchsin and leukocytes with an H&E counterstain. Islets (at least 20/mouse in most cases) were individually scored as follows: 0, no lesions; 1, peri-insular leukocytic aggregates, usually periductal infiltrates but no islet destruction; 2, ≤25% islet destruction; 3, >25–75% islet destruction; and 4, >75% islet destruction. An insulitis index was calculated by the formula: insulitis index = (total score for all islets)/4 (number of islets examined). Diabetic mice or animals that died after becoming diabetic were assigned an insulitis index of 1, because these mice usually had <20 remaining islets and all examined islets were score 4.

Peptides and tetramers

IGRP06–214 (VYLLKTNVL), Ins-19 (LYLVCGERL), NRP-V7 (KYN KANVFIL), and MimA2 (YAIENYLEL) peptides were synthesized by standard solid-phase methods using F-moc chemistry in an automated peptider synthesizer (model 435A; Applied Biosystems) and their identities were confirmed by mass spectrometry. Concentrated stocks (10 mM) were prepared in DMSO and 10 μM working stocks were obtained by dilution in PBS. PE-labeled H-2Kb tetramers loaded with NRP-V7 or Ins19 peptides were produced as described previously (18). PE-labeled H-2Db tetramers loaded with MimA2 peptide were produced by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Tetramer Core Facility. Soluble native murine and human CD1d proteins were prepared using baculovirus and mammalian cell expression systems and enzymatically biotinylated at a specific C-terminal biotin ligase site as described in previous reports from our laboratories (8, 19). Biotinylated CD1d proteins at 2 μM were incubated with a 20-fold excess of the indicated glycolipid for 24 h and subsequently assembled into tetramers by addition of 0.5 μM allophycocyanin- or PE-conjugated streptavidin. To produce fluorescent glycolipid-loaded CD1d dimers or monomers, 2 μM biotinylated murine CD1d/glycolipid complexes were mixed with biotinylated human CD1b protein (produced by expression in Chinese hamster ovary cells as previously published (19)) at a ratio of 1:1 (for dimers) or 3:1 (for monomers), and then incubated with 0.5 μM PE-conjugated streptavidin.

Propagation of islet-infiltrating T cells
Islet isolation by collagenase perfusion of the common bile duct was modified from a previously described protocol (18). Briefly, the bile duct was cannulated and the pancreas perfused with collagenase P (Roche). The inflated pancreas was removed and incubated at 37°C to digest exocrine tissue. Following dispersion of digested tissue and three washes with HBSS, islets were resuspended in HBSS containing DNPase I (Worthington Biochemical) and a handpicked using a siliconized micropipet under a dissecting microscope (all islets that could be identified were collected and enumerated separately for each mouse). Isolated islets were washed with 2% FBS in HBSS, resuspended in RPMI 1640 medium supplemented with 10% FBS (HyClone), and 50 μM recombinant human IL-2 (PeproTech), and cultured in 24-well tissue culture plates (~30 islets/well) at 37°C, 5% CO2. Cultures were harvested after 7 days, and cells were cryopreserved in aliquots for subsequent FACS and ELISPOT analyses.

Preparation of cell suspensions from mouse tissues
For thymocyte suspensions, aseptically removed thymuses from 6- to 8-wk-old C57BL/6 mice were mechanically forced through stainless steel mesh, and tissue fragments were removed by settling. Cell suspensions were prepared from spleens and pancreatic lymph nodes (PLN) by gently grinding the tissues with a syringe plunger on the surface of a 70-μm nylon mesh, and cells were washed through and collected with PBS. For dendritic cell (DC) isolation, spleen and PLN were first injected with a solution of collagenase at 400 U/ml (Sigma-Aldrich), cut into small pieces, and suspended in 100 U/ml collagenase, then incubated for 4 h at 37°C. Tissue fragments were harvested and disaggregated into single-cell suspensions by passing through nylon mesh.

Measurement of cytokine secretion by in vitro- and in-vivo-activated iNKT cells
For in vitro activation of murine splenic inNKT cells, splenocytes from BALB/c mice were plated at 5 × 107 cells/well in 96-well flat-bottom tissue culture plates and stimulated with αGalCer analogs at a range of concentrations. Culture supernatants were harvested after 48 h at 37°C and levels of IL-4, IL-13, and IFN-γ were quantified by ELISA. For in vitro activation, murine iNKT hybridomas or human iNKT cell clones at 5 × 104/well were stimulated with 5 × 105/μl murine CD1d-transfected A20 cells or human DCs (derived by in vitro culture of peripheral blood monocytes for 3 days in medium supplemented with GM-CSF and IL-4) in the presence of the indicated concentrations of αGalCer analogs. Levels of murine IL-2 were measured at 12 h, and levels of human IL-12, IL-13, IFN-γ, and TNF-α were measured at 48 h in culture supernatants by capture ELISA. For in vivo iNKT cell activation, female NOD or C57BL/6 mice, 5–8 wk of age, were injected i.p. with vehicle or αGalCer analogs (4 μg/mouse). Blood was collected 2, 6, and 21 h postinjection by retro-orbital puncture and serum samples were diluted 1:5 with PBS and tested for IL-4, IFN-γ, and IL-12p70 by ELISA. Cytokine production by iNKT cells was assessed 2 h postinjection by intracellular IL-4 and IFN-γ cytokine staining followed by FACS analysis. All ELISA used specific capture and biotinylated detection mAb pairs (BD Pharmingen), streptavidin-alkaline phosphatase (Zymed Laboratories) and 4-nitrophenyl phosphate (Sigma-Aldrich) as substrate. Recombinant cytokines (PeproTech) were used to generate standard curves. Measurements were performed in duplicate or triplicate.

IFN-γ ELISPOT assays
ELISPOT plates (MAHA S45 10; Millipore) were precoated with anti-murine IFN-γ mAb (R4-6A2; BD Pharmingen) and blocked with 1% BSA (Fraction V; Sigma-Aldrich) in PBS. Mitomycin C-treated RMA-S/Kb cells were added at 2 × 104 cells/well and pulsed with 1 μM peptide.
Flow cytometry

Single-cell suspensions were stained in FACS buffer (1% BSA plus 0.05% NaN₃ in PBS) with PE- or allophycocyanin-conjugated glycolipid-loaded CD1d tetramers for 1 h at room temperature, followed by staining when indicated with fluorescent-labeled Abs for 30 min at 4°C. For equilibrium binding assays using CD1d tetramers, dimers, or monomers, C57BL/6 thy-mocytes or iNKT hybridoma cell lines were incubated with the indicated concentrations of PE-labeled CD1d complexes in 50 μl of FACS buffer for 1 h at room temperature. For intracellular cytokine staining, cells were stained with allophycocyanin-conjugated αGalCer-CD1d tetramers for 3 h on ice before being permeabilized using a BD Pharmingen Cytofix/Cyto-perm kit, and then stained for 30 min at 4°C with the relevant PE-conju-gated anti-cytokine Ab. Nonspecific staining was blocked using 10 μg/ml rat anti-mouse CD16/32 (2.4G2; BD Pharmingen). For analysis of in vitro-expanded T cell cultures, aliquots of cryopreserved cells were thawed and incubated with FITC-conjugated anti-murine CD8α mAb, allophycocyanin-conjugated anti-murine CD4 mAb and PE-labeled peptide-loaded H-2Kd or H-2Db tetramers in the presence of mAb 2.4G2. FITC-, PE-, PerCP- or allophycocyanin-conjugated Abs to TCRαβ, B220, CD44, CD69, CD25, CD4, CD8α, CD11c, CD80, CD86, I-A^d (cross-reactive with I-A^k), IL-4, and IFN-γ were all purchased from BD Pharmingen. Samples were analyzed by flow cytometry using a FACSCalibur instru-ment and CellQuest software (BD Immunocytometry Systems).

Statistics

GraphPad Prism software was used for statistical calculations and p values <0.05 were considered significant. One-way ANOVA was used for analysis of experiments with three or more data sets, and posttest comparisons were analyzed by either unpaired Student’s t test or Mann-Whitney non-parametric test as indicated. Differences in Kaplan-Meier curves for onset of glycosuria and death were analyzed by log-rank test. For analysis of insulitis index (see Fig. 4B), a weighted least squares linear regression was used to test for significance. To prevent overrepresentation by animals that survived longer as a result of random background resistance to develop-ment of diabetes (e.g., not all NOD mice develop diabetes), data from surviving animals was supplemented with imputed insulitis scores of 1.0 for all animals that died before an analysis time point. With the supple-mented data, linear regression analysis was conducted with the insulitis score as the outcome variable. The predictor variables were indicators for week, treatment group, and their interaction effects. To reduce hetero-cedasticity, the regression analysis weighted each observation in proportion to the number of islets assessed in that animal. Because islets of deceased animals could not be assessed, the imputed insulitis scores for these ani-mals were weighted as if three islets had been observed, which was the median number of islets assessed per animal for all experimental groups combined.

Results

Stimulation of altered cytokine responses by αGalCer analogs in vitro and in vivo

Analogos of αGalCer KRN7000 with fatty acid chain substitutions (C20:2 and C24:0) were identified by our earlier studies as promising candidates for further development as in vivo iNKT cell ago-nists (Fig. 1A) (8). To assess further the potential applications of these novel compounds, we compared their iNKT cell-activating properties to those of two other well-studied forms of αGalCer, KRN7000 and OCH. Using a panel of mouse iNKT cell hybrid-omas expressing different TCR Vβ chain segments, we confirmed the stimulatory properties of all four αGalCer analogs. However, there were substantial variations in potency for the four com-pounds, with C26:0 consistently showing the most potent activity while OCH had the weakest (Fig. 1A). The OCH and C20:2 com-pounds have previously been described as stimulators of a Th2-biased iNKT cell response (6, 8) and we confirmed and extended this observation in studies of in vitro mouse splenocyte stimulation that compared the production of two Th2-associated cytokines (IL-4 and IL-13) with the concurrent production of IFN-γ (Fig. 1B). This analysis revealed that C20:2 was a particularly strong inducer of both IL-4 and IL-13 (Fig. 1B, left). By considering the ratios of IL-4 or IL-13 to IFN-γ production, it was apparent that these were substantially higher over the entire range of glycolipid concentrations for both C20:2 and OCH compared with C24:0 and C26:0 (Fig. 1B, right). These findings confirmed the previous charac-terization of C20:2 and OCH as Th2-biasing αGalCer analogs and the direct comparison of these two analogs indicated that C20:2 had the potential to stimulate substantially greater quantities of Th2 cytokines.

To compare in vivo the relative cytokine stimulation by the four αGalCer analogs, we assessed the serum levels of IL-4, IFN-γ, and IL-12p70 after i.p. injection of a single dose of each glycolipid. IL-4 levels are known to peak at 2 h after αGalCer stimulation in this assay, whereas IFN-γ peaks much later and reaches a plateau between 20 and 24 h (6). The levels of IL-4 and IFN-γ observed at their expected peak time points were supportive of the previous characteriza-tion of C20:2 and OCH as Th2-biasing analogs (Fig. 1C). Again, the direct comparison suggested a more potent stim-ulation of IL-4 by C20:2, although this trend did not reach statisti-cal significance (p = 0.06, one-way ANOVA). Interestingly, IL-12p70 levels did not show any significant variation between the four compounds, as all of them showed a peak of similar magni-tude at 6 h (Fig. 1C), followed by a return to undetectable levels by 20 h (data not shown). This finding underscores the complexity of the iNKT cell cytokine response, which even following stimulation with C20:2 or OCH shows certain features that are typically linked with Th1-type inflammatory responses. Furthermore, these find-ings indicate that the failure of C20:2 and OCH to stimulate a strong and sustained IFN-γ response cannot be attributed to the failure of these analogs to evoke IL-12p70 release, which is in contradiction to a previous report describing OCH as a less potent inducer of IL-12p70 than KRN7000 (15).

High-avidity interaction of αGalCer C20:2 with TCRs of murine and human iNKT cells

Because avidity of the TCRs of iNKT cells with their specific ligands has been proposed as an important determinant of the out-come of activation (20, 21), we assessed this parameter for each of the four αGalCer analogs. First, we used costaining of murine splenocytes with allophycocyanin-labeled murine CD1d tetramers loaded with C26:0 plus PE-labeled murine CD1d tetramers loaded with each of the different analogs to determine whether all of these were recognized by the same iNKT cell populations. This proved to be the case, as each analog-loaded tetramer bound to all C26:0-loaded tetramer-positive T cells, albeit with varying intensities (Fig. 2A, left). We then measured the equilibrium-binding levels of murine CD1d tetramers loaded with each of the αGalCer analogs over a range of tetramer concentrations to determine the equilib-rium dissociation constant for tetramer binding (K_D), which is in-versely related to TCR avidity (Fig. 2B, center). Analogous bind-ing curves and K_D values were similarly generated using αGalCer analog-loaded dimers and monomers (Fig. 2A, right). The K_D values for iNKT cell TCR interactions with either multivalent or monovalent ligands were low for C20:2-loaded CD1d complexes, indicating a binding strength similar or even greater than that for complexes of CD1d with the non-Th2-skewing analogs, C26:0 and C24:0. In contrast, OCH-loaded CD1d complexes showed much higher K_D values, consistent with relatively weak binding to the TCRs of mouse iNKT cells.
Previous studies have emphasized the strong conservation of CD1d and iNKT cell responses between mice and humans, which supports the use of mouse models in preclinical exploration of iNKT cell-based therapies (22). Nevertheless, the surprisingly weak interaction of OCH with TCRs of mouse iNKT cells prompted us to examine whether this analog could be recognized significantly by human iNKT cells. This was analyzed initially by using human CD1d tetramers loaded with each of the four \( /H9251 \) GalCer analogs to stain human peripheral blood iNKT cells. Whereas all cells that stained with C26:0-loaded human CD1d tetramers were strongly costained by C24:0- or C20:2-loaded human CD1d tetramers, we could not detect any significant staining with OCH-loaded human CD1d tetramers in peripheral blood of normal human subjects (Fig. 2B, left). We conducted similar tetramer staining studies on a panel of in vitro-expanded human iNKT cell clones (clones DN2.D5, DN2.C4, DN2.C5, HDD.11, HDA.A7, and HDA.A8) and again failed to detect significant staining with OCH-loaded human CD1d tetramers while tetramers loaded with the other \( /H9251 \) GalCer analogs stained strongly (Fig. 2B, left, and data not shown). The tetramer binding results were confirmed by functional responses of human iNKT cell clone DN2.D5 (Fig. 2B, right) showing that C20:2, C26:0, and C24:0 were all strong activators of both Th2 and Th1 associated cytokine production, whereas OCH failed to stimulate any detectable responses.

Induction of Th2-biased cytokine response by C20:2 in NOD mice

Although a previous study found that repeated administration of OCH was effective for prevention of type 1 diabetes in NOD mice (12), our finding that OCH had no stimulatory activity for human iNKT cells indicated that this compound may not be suitable for development as an immunomodulator for use in humans. Given the strong activity of the C20:2 analog on human iNKT cells, it was of interest to know whether this analog would also behave as a strong iNKT cell activator and give preferential Th2-cytokine induction in NOD mice. Examining this question first in vitro, we found that C20:2 induced NOD splenocyte cultures to produce significantly more IL-4 and less IFN-\( \gamma \) compared with C24:0 (Fig. 3A, top panel). In vivo, we found that the rapid burst of serum IL-4 occurring 2 h after injection was significantly higher in C20:2-treated mice compared with C24:0, whereas the delayed production of IFN-\( \gamma \) usually observed at 20–24 h postinjection was absent in C20:2-treated mice (Fig. 3A, bottom panel). The iNKT cell responses of NOD mice were overall significantly weaker than...
those previously observed in C57BL/6 mice (8), consistent with the well-described quantitative and qualitative deficiencies of iNKT cells characteristic of NOD mice (2). We also used intracellular cytokine staining and FACS to examine more directly the impact of C20:2 stimulation on iNKT cell cytokine production (Fig. 3B). This showed that in splenocytes, C20:2 was not able to induce a Th2 bias at the iNKT cell level because similar percentages of iNKT cells secreting IL-4 and IFN-\(\gamma\) were obtained in C20:2- and C24:0-treated mice, respectively. However, in PLN, C20:2 triggered a significant increase in the percentage of IL-4-positive iNKT cells compared with either vehicle or C24:0-treated mice, and this was associated with low percentages of IFN-\(\gamma\)-positive iNKT cells (Fig. 3B).

**FIGURE 2.** Molecular interactions of \(\alpha\)GalCer analogs with TCRs of murine and human iNKT cells. A, Mouse CD1d tetramers labeled with allophycocyanin were loaded with each of the glycolipid analogs (x-axis; glycolipid used for loading is indicated in box surrounding each dot plot), and used together with PE-labeled C26:0-loaded mCD1d tetramers (y-axis) to costain C57BL/6 thymocytes (left). Tetramers loaded with each of the analogs were titrated for staining of mouse iNKT cell hybridoma cells (DN3A4-1.2) to generate equilibrium binding curves based on measurement of mean fluorescence intensity (MFI) by FACS (middle). This procedure was also conducted with fluorescent dimers and monomers of mCD1d loaded with each glycolipid, and equilibrium dissociation constants (\(K_D\)) were determined as the concentrations of CD1d/glycolipid complexes required to give 50% of maximal binding (right). B, Similar analyses as described for murine iNKT cell binding of glycolipid tetramers in A were conducted using human CD1d tetramers to stain human PBL (dot plots), or a human \(\alpha\)24+ iNKT cell clone, DN2.D5 (histograms; heavy line is staining with tetramer loaded with indicated glycolipid, and light line is unloaded tetramer control) (left). Cytokine secretion by iNKT cell clone DN2.D5 cultured for 48 h with human monocyte-derived DCs and each of the glycolipids was determined using capture ELISAs for IL-4, IL-13, IFN-\(\gamma\), and TNF-\(\alpha\) (right). Stimulation with immobilized anti-CD3 mAb (OKT3) was used as a positive control for T cell activation. * Below limits of detection (<0.05 ng/ml).

**FIGURE 3.** Cytokine responses of NOD iNKT cells to \(\alpha\)GalCer analogs. A, Splenocytes from female NOD mice (5 wk of age) were stimulated with 100 nM C20:2 or C24:0 \(\alpha\)GalCer for 72 h and supernatants were tested for IL-4 and IFN-\(\gamma\) content by ELISA (top). Bars represent means of triplicates cultures. Serum levels of IL-4 and IFN-\(\gamma\) in mice given 4 \(\mu\)g i.p. of C24:0 or C20:2 were measured by ELISA at 2 and 23 h postinjection (bottom). Bars show means and SDs for groups of five mice. One representative example of two separate experiments is shown. B, Female NOD mice (5 wk old) received a single i.p. injection of either inert vehicle or 4 \(\mu\)g of glycolipid (C20:2 or C24:0), and 2 h later the spleens and PLNs were harvested for intracellular staining of IL-4 or IFN-\(\gamma\). Means and SDs of triplicate measurements are shown. * \(p < 0.01\) (upper panel) or \(p < 0.05\) (lower panel) compared with vehicle control (unpaired Student’s t test).
Efficacy of C20:2 for diabetes prevention and inhibition of progressive insulitis

Having established that C20:2 evoked a Th2-biased cytokine profile in NOD mice, we investigated the effect of this compound on diabetes incidence by comparing its effects directly to those of the C24:0 analog which showed similar potency for iNKT cell activation but a more mixed cytokine response. Female NOD mice were treated starting from 4 to 6 wk of age with weekly i.p. injections of αGalCer analogs for 7 wk and progression of diabetes was monitored by development of glucosuria and time to death (Fig. 4A). Although both iNKT cell activators gave a significant delay in diabetes onset and death compared with vehicle-treated animals, the C20:2 analog showed significant improvement in both outcomes compared with C24:0. This was evident both in the median time to development of glucosuria (36 wk for C20:2 vs 27 wk for C24:0) and in the median survival (>50 wk for C20:2 vs 40 wk for C24:0).

Because the two αGalCer analogs had a different impact on diabetes development in NOD mice, we next compared their effect on the progression of insulitis with age. As shown in Fig. 4B, mice treated with C24:0 showed significantly reduced insulitis at 17 wk of age (i.e., 4–6 wk after cessation of treatment) compared with vehicle- and C20:2-treated animals. However, insulitis progressed with age in C24:0-treated groups, ultimately reaching the same severity as the vehicle-treated control animals at 53 wk of age. In contrast, treatment with C20:2 did not give detectable inhibition of insulitis at 17 wk of age, but did show a significant reduction of insulitis in animals at 53 wk of age (Fig. 4B). This observation suggested that at least part of the protective effect with C20:2 involved different mechanisms than with C24:0 treatment, leading to more pronounced suppressive effects on the long-term progression of late insulitic lesions.
Systemic and local alterations in T cell subsets following treatment with αGalCer analogs

Our regimen of seven weekly injections of αGalCer was based on earlier published observations suggesting that effective prevention of diabetes in NOD mice requires multiple injections of the iNKT cell agonist (3–5, 12). Although in vivo dynamics of iNKT cells after a single injection of αGalCer are well-described (23), the outcome of responses to serial injections of iNKT cell ligands are more complex and may lead to an exaggerated contraction of cell numbers and energy (24–26). To determine the impact of our multiple injection regimen on iNKT cells in NOD mice, we monitored their levels and phenotype in the spleen and PLN at different intervals after receiving a course of seven weekly injections of either C20:2 or C24:0 (Fig. 5).

In spleen, the percentages of iNKT cells in both C20:2- and C24:0-treated animals were significantly reduced compared with vehicle-treated animals, from 1 wk up to 30 wk after the last injection (Fig. 5A, left). Although the kinetics were slower, a similar phenomenon was detected in PLN of treated mice (Fig. 5A, right). No expansion of iNKT cells was observed in either of the αGalCer-treated groups, and iNKT cell levels remained significantly reduced even at 30 wk after the cessation of treatment, indicating a long-lasting depletion of iNKT cells rather than a temporary down-regulation of their TCRs. Phenotypic studies showed that the minor fraction of iNKT cells that was resistant to depletion in the spleen at 1 wk following the last injection of αGalCer analogs had down-regulated the activation markers CD25, CD44, and CD69 (Fig. 5B), suggesting a hyporesponsive or partially anergic state. Similar results were observed in spleen and PLN at 1, 5, and 27 wk posttreatment (data not shown), indicating that this was a durable and possibly even irreversible phenomenon.

The slowing of the late progression of insulitis that we observed in C20:2-treated mice (Fig. 4B) suggested that treatment with this analog might have reduced the expansion or homing of islet β cell autoantigen-reactive CD4+ or CD8+ T cells to the pancreatic islets. To explore this possibility, we isolated pancreatic islets from...
vehicle-treated and C20:2-treated NOD mice 4 wk after cessation of treatment (i.e., 15 wk of age), and expanded the islet-infiltrating T cells by short-term culture in medium containing IL-2. Total numbers of both CD4\(^+\) and CD8\(^+\) T cells recovered were markedly reduced in the C20:2-treated animals compared with controls, whether this was calculated on a per mouse or per islet basis (Fig. 6A). To analyze specifically the \(\beta\) cell-reactive T cells, we took advantage of some of the known specificities of islet-reactive CD8\(^+\) T cells corresponding to peptides derived from insulin B chain, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and dystrophia myotonica kinase (18, 27, 28). Both ELISPOT and MHC class I tetramer analyses were used to examine the frequencies of these autoreactive CD8\(^+\) T cells in islet-infiltrating T cell populations derived from vehicle-treated and C20:2-treated NOD mice (Fig. 6, C and D). Consistent with other studies, we found that T cells reactive with IGRP peptide 206–214, or its mimotope NRP-V7, were the most prevalent among the defined populations of autoantigen-specific CD8\(^+\) T cells (18, 28). Although the relative frequencies of CD8\(^+\) T cells reactive with the three autoantigenic peptides did not differ significantly between islet-derived T cell populations from control and C20:2-treated animals, there was a substantial reduction in the total number of CD8\(^+\) T cells reactive with the three defined islet autoantigenes (Fig. 6, C and D). This reduction in autoantigen-specific CD8\(^+\) T cells was demonstrated using both tetramer staining and IFN-\(\gamma\) ELISPOT and was of similar magnitude whether quantitated on a per mouse or per islet basis.

**Effects of \(\alpha\)GalCer treatment on DC subset and maturation anomalies in NOD mice**

Previous studies have suggested that activation of iNKT cells may suppress autoimmunity in NOD mice at least partly through effects on frequency and functions of tolerogenic DCs in the PLNs (29, 30). To assess the effects of \(\alpha\)GalCer analog treatment on DCs during diabetes progression, we monitored the levels in spleen and PLN of various DC subsets, including conventional myeloid CD11chighCD8\(^-\), CD11chighCD8\(^+\), and plasmacytoid CD11clow DC, during the course of the disease in treated and untreated NOD mice. As shown in Fig. 7A, we observed an alteration in the relative proportions of these three DC subsets in both spleen and PLN of control (vehicle-treated) NOD mice as these animals aged from 16 to 30 wk. Specifically, we observed that diabetes progression was accompanied by a significant augmentation of the proportion of plasmacytoid CD11clow DC, most of which were B220\(^{low}\) (data not shown), and a significant reduction of the proportion of myeloid CD11chighCD8\(^-\) DCs (Fig. 7A). Interestingly, treatment of NOD mice during the prediabetic phase with \(\alpha\)GalCer analogs C24:0 or C20:2 significantly reduced the evolution of DC subset alterations observed in the vehicle-treated animals at 30 wk, and restored the relative proportions of the three DC subsets to levels that approximated those present in 16 wk old prediabetic mice (Fig. 7B). This effect was observed in the PLNs of both C24:0- and C20:2-treated mice, although a trend toward greater correction of the DC imbalance was observed in the C20:2-treated animals (Fig. 7B). No posttreatment modifications were observed in splenic DCs from the same mice, indicating that \(\alpha\)GalCer treatment specifically targeted the DC abnormality in PLNs.

Knowing that NOD mice display defective maturation of myeloid DCs (29, 31), we also evaluated the alteration of DC surface phenotype after treatment with C20:2 and C24:0. Using a single i.p. injection, we found that both forms of \(\alpha\)GalCer were able to induce similar maturation of spleen and PLN CD11chigh DCs in prediabetic NOD mice within 24 h, as characterized by up-regulation of MHC class II and costimulatory molecules (CD86, CD80,
and CD40) (Fig. 8A and additional data not shown), as has been described previously for C57BL/6 mice (32). The DC maturation was transient, as demonstrated by a return of all maturation markers to their baseline levels by 3 days after stimulation (data not shown). We next compared the maturation phenotype of CD11c<sup>bright</sup> DCs in spleen and PLN at different time points after seven weekly injections of either vehicle, C20:2 or C24:0. One day after the last of the seven injections, quantitation of MHC class II, CD80, and CD86 expression by DCs indicated the full acquisition of a maturation phenotype both in spleen and PLN (data not shown) that was not appreciably different from the DC profile obtained after a single injection (Fig. 8A). In contrast, the DCs of 30-wk-old animals (i.e., 17–19 wk after the last injection) showed persistent alterations in maturation phenotype, including up-regulation of MHC class II expression in the spleens of both C24:0- and C20:2-treated mice (Fig. 8B, left), and significant down-regulation of MHC class II and CD80 levels in the PLNs of C20:2-treated mice (Fig. 8B, right).

**Discussion**

Recent studies have revealed the ability to elicit more selective expression of iNKT cell functions using chemically modified forms of αGalCer (6, 8), although little information is currently available on potential compounds to the prevention or treatment of specific diseases. In the current study, we have analyzed multiple clinical, pathological, and immunological outcomes in NOD mice to evaluate the impact of αGalCer C20:2, a novel synthetic Th2-cytokine skewing iNKT cell agonist developed in our laboratories, on autoimmune diabetes. Our initial analysis of the iNKT cell-activating properties of C20:2 demonstrated several important potential advantages over other previously studied αGalCer analogs. Although αGalCer sphingosine and αGalCer C20:2 stimulate cytokine production by both iNKT cells and other T cells, αGalCer C20:2 was a potent ligand for human iNKT cells, while αGalCer sphingosine analogs of C20:2 were not. This leads to the conclusion that OCH and possibly other truncated sphingosine analogs of αGalCer may not be useful as immunomodulators in humans, whereas N-acyl derivatives such as C20:2 could prove to be much more promising. It should be noted that the absence of Th2 cytokine-biased responses to C20:2 by human iNKT cell clones that we observed (Fig. 2B) is expected, because studies in mice show that this cytokine bias is mainly due to the failure of C20:2 to induce the ability of iNKT cells to transactivate Th1 cytokine production by other leukocytes such as NK cells (8). Thus, the potential for inducing a Th2 cytokine bias can only be evaluated using in vitro systems that allow stimulation of complex cell mixtures such as whole splenocytes, or by in vivo activation of iNKT cells within the context of an intact host.

We also showed that the C20:2 analog was more effective for prevention of diabetes in NOD mice when compared with a related αGalCer analog (C24:0) which had similar potency for iNKT cell activation but did not stimulate a Th2 cytokine bias. The analysis of diabetes prevention reported in this study differs from previous studies in a number of important ways. First, we extended the period of observation beyond that which has been previously reported (32 wk or less), and observed animals through >45 wk to demonstrate evidence of sustained, long-term benefit. Second, we initiated therapy in most of our experiments at 4–6 wk of age, as opposed to nearly all previous studies of this type which initiated therapy with KRN7000 or OCH in 3- to 4-wk-old female NOD mice sacrificed 24 h following one i.p. injection of either inert vehicle or 4 μg of C20:2. Representative FACS histograms show cell surface levels of the indicated proteins (x-axis) vs cell number (y-axis) on DCs gated according to forward and side light scatter and for high CD11c expression. Shaded histograms are DCs from PLN of vehicle-treated control mice, and open histograms with bold outline DCs from PLN of C20:2-treated mice. Results shown are representative of those obtained with five mice in each group. Similar results were obtained for splenic DCs (data not shown). B, Expression of DC maturation markers on CD11c<sup>bright</sup> cells in spleen (left) or PLN (right) of 30-wk-old NOD female mice treated from age 4 wk with seven weekly i.p. injections of either vehicle (○), or 4 μg of αGalCer analogs (C24:0 (■) or C20:2 (●)). Bars represent means and SDs of the MFI values determined by FACS for five mice in each treatment group. * Significant differences (p < 0.05, Mann-Whitney U test) compared with vehicle control group.
mice (3–5, 12, 30). Finally, our treatment protocol was also significantly less intensive in terms of frequency and duration of αGalCer administration, consisting of injections once per week for 7 wk as compared with daily or twice weekly injections for much longer than 7 wk in most published studies (3–5, 12, 30). In our view, this later initiation of treatment and less intensive regimen may more closely resemble an intervention that could be feasible in actual clinical settings, following as a model the encouraging example of beneficial sustained immunomodulation in humans with new onset diabetes using short course i.v. administration of modified anti-CD3 mAb (33).

A further point that supports the use of a less intensive regimen for iNKT cell activation is the known ability of repeated injections of KRN7000 in mice to induce a form of hyperresponsiveness or partial anergy in these T cells (24, 25). This has been associated with reduced proliferative capacity to subsequent stimulation and also with diminished cytokine secretion. In our study, we observed a very substantial reduction in iNKT cell numbers following seven weekly injections of αGalCer. This contraction of the iNKT cell population was sustained for at least 30 wk and preliminary studies suggest that it may never completely resolve during the lifespan of the animals (C. Forestier and S. A. Porcelli, unpublished data). Interestingly, in vehicle-treated NOD mice, we observed a reduction in the percentage of iNKT cells in the spleen between 12 and 17 wk of age, which was accompanied by a reciprocal rise in the percentage of iNKT cells in the PLNs of these animals during the same time period. This may have reflected an increased migration of iNKT cells during this period into the PLNs and possibly other tissues involved in the autoimmune inflammatory process. These time-dependent changes in iNKT cell numbers were significantly blunted in C20:2- and C24:0-treated animals, possibly because of a partial depletion of iNKT cells resulting from the treatment. These findings raise issues about the potential long-term implications of aggressive treatment with αGalCer and suggests that further investigations will be needed to identify the optimal approach to activating the regulatory properties of iNKT cells without leading to their prolonged or permanent depletion.

The primary mechanism by which αGalCer treatment suppresses autoimmunity in NOD mice remains a contentious issue. Initial work strongly suggested that the creation of a Th2-promoting environment, presumably due to the secretion of IL-4 and possibly other Th2-biasing cytokines by activated iNKT cells, was responsible for the beneficial effects on disease. Indeed, it has been reported that KRN7000 treatment of NOD mice leads to a Th2 cytokine-enriched environment in the spleen and PLN, and CD4+ T cells specific for islet autoantigens show enhanced Th2 cytokine production in these mice (3, 4). The relevance of this mechanism is supported by a report showing that suppression of diabetes by αGalCer is largely dependent on IL-4 (34), although this appears not to have been confirmed by all investigators (29). Our observations in the current study of improved diabetes suppression with the Th2-skewing C20:2 analog are consistent with an important role for Th2 cytokines such as IL-4 or IL-13 in the mechanism of disease prevention, as is the finding of improved outcomes in this model following treatment with the OCH analog (12).

An alternative mechanism for the action of αGalCer in diabetes prevention is the indirect effect of this compound in inducing the maturation and migration of DCs (29, 30). It is well-known that activation of iNKT cells leads to DC maturation through the actions of costimulatory molecules and cytokines, such as CD40L and IL-12 (32, 35). This may potentially overcome the demonstrable defect in DC maturation in NOD mice (36) and in humans with type 1 diabetes (37). A recent publication has highlighted the induction of regulatory or tolerogenic DCs following repeated induction of αGalCer into normal mice (38) and these appear to have predominantly a CD11c<sup>high</sup>CD8α<sup>−</sup> phenotype. This is consistent with the DC subset that has been previously found to be tolerogenic in NOD mice (39), and that was previously shown to be recruited to the PLN by αGalCer treatment (30). This latter finding was confirmed in the current study, which demonstrated the effect of αGalCer in maintaining the relative proportions of DC subsets in the PLNs of 30-wk-old NOD mice at levels similar to those seen in younger animals that lack clinically apparent autoimmune disease. Another interesting finding from our analyses was that DCs in the PLN of C20:2-treated mice showed persistent down-regulation of MHC class II and CD80, raising the possibility that αGalCer-mediated protection might be associated with the establishment of an anergic state in DCs at this key location.

A third mechanism to explain the effect of αGalCer on ameliorating autoimmunity in NOD mice is the impact of activated iNKT cells on other T cell subsets that either mediate or regulate autoimmune insults. For example, the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3-dependent regulatory T cell (Treg) population has recently been found to engage in significant cross-talk with iNKT cells (40). In preliminary studies, we have not observed any gross alterations in the levels of the total CD4<sup>+</sup>CD25<sup>+</sup> T cell populations in either spleen or PLN following αGalCer treatment of NOD mice (data not shown). However, while we have not studied the levels of Foxp3<sup>+</sup> T cells in treated vs untreated animals and it remains unclear whether numbers or functions of Tregs specific for islet Ags are altered by treatment with iNKT cell activators. In contrast, the current study did provide clear evidence for a marked influence of iNKT cell activation on the autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> effector T cell populations in the pancreatic islets of NOD mice. Although we did not evaluate in detail the effects on CD4<sup>+</sup> autoreactive effector T cells in our studies, we observed a marked reduction in total CD4<sup>+</sup> T cells in the T cell populations expanded from pancreatic islets of C20:2-treated NOD mice. More notably, our detailed analysis of CD8<sup>+</sup> T cells specific for defined autoantigens showed these to be markedly reduced in frequency in C20:2-treated mice. To our knowledge, this is the first study to assess the impact of iNKT cell activation with αGalCer on the frequency of natural nontransgenic CD8<sup>+</sup> T cells with defined β cell autoantigen specificities in the pancreatic islet infiltrates. Our studies have not yet compared the relative efficacy of C20:2 with non-Th2-biasing αGalCer analogs with regard to the impact on autoreactive CD8<sup>+</sup> T cells in the islets, which will be an interesting focus for detailed future studies in this area.

In summary, we have confirmed the potential regulatory role of iNKT cells in type 1 diabetes and provide further support for the development of novel αGalCer compounds as therapeutic immunomodulators for prevention and treatment of this disease. Our findings also emphasize the beneficial influence of a Th2 cytokine environment in diabetes-prone animals, which was apparent from the improved clinical and immunological outcomes achieved with the Th2 cytokine-skewing C20:2 analog. Based on the results that we have obtained to date comparing C20:2 with OCH, the other well-studied αGalCer analog known to generate Th2 cytokine-biased responses, we propose that C20:2 is a more promising potential therapeutic agent because of its high avidity for the TCRs of human iNKT cells. Our studies have not yet assessed whether the Th2-skewing property of C20:2 is conserved in other mammals besides mice, particularly nonhuman primates and humans, which will be an important next step in determining the potential of C20:2 as an agent for prevention or therapy of diabetes or other human autoimmune diseases.


