A Single Early Activation of Invariant NK T Cells Confers Long-Term Protection against Collagen-Induced Arthritis in a Ligand-Specific Manner


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A Single Early Activation of Invariant NK T Cells Confers Long-Term Protection against Collagen-Induced Arthritis in a Ligand-Specific Manner


The glycosphingolipid α-galactosylceramide (α-GalCer) has been shown to be a potent activator of invariant NKT (iNKT) cells, rapidly inducing large amounts of both Th1 and Th2 cytokines upon injection in mice. The C-glycoside analog of α-GalCer (α-C-GalCer), by contrast, results in an enhanced Th1-type response upon activation of iNKT cells. We administered a single dose of these Ags to DBA/1 mice during the early induction phase of collagen-induced arthritis and demonstrated therapeutic efficacy of α-GalCer when administered early rather than late during the disease. Surprisingly, the Th1-polarizing analog α-C-GalCer early during the course of disease resulted in partial improvement of clinical arthritis symptoms, blockade of IFN-γ release later on resulted in a more rapid onset of arthritis. Although no phenotypic changes in conventional T cells, macrophages, or APCs could be detected, important functional differences in T cell cytokine production in serum were observed upon polyclonal T cell activation, 2 wk after onset of arthritis. Whereas α-GalCer-treated mice produced significantly higher amounts of IL-10 upon systemic anti-CD3 stimulation compared with PBS controls, T cells from α-C-GalCer-treated mice, by contrast, produced substantially lower levels of cytokines, suggesting the involvement of different protective mechanisms. In conclusion, these findings suggest long-term, ligand-specific, time-dependent, and partially IFN-γ-dependent immunomodulatory effects of iNKT cells in collagen-induced arthritis. The Journal of Immunology, 2007, 179: 2300–2309.

Natura l killer T (NKT) cells constitute a unique population of lymphocytes that coexpress receptors of the NK cell lineage and intermediate levels of TCRβ. The majority of NKT cells in mice express an invariant TCRα chain, encoded by a Vα14-Jα18 rearrangement, and a restricted TCR Vβ repertoire. Therefore, these cells are often referred to as invariant NKT (iNKT)4 cells (1, 2). Unlike conventional T cells, the vast majority of mouse iNKT cells recognize glycolipid Ags, including α-galactosylceramide (α-GalCer), a glycosphingolipid originally isolated from marine sponges. These glycolipids are presented to iNKT cells by a nonpolymorphic MHC class I-like Ag-presenting molecule, CD1d (3–5). A characteristic feature of iNKT cells is their rapid production of both Th1- and Th2-type cytokines upon activation (3, 6–8). These cytokines originate either from iNKT cells or from downstream effector cells such as NK cells or conventional T cells (9–11). In humans, a similar iNKT cell subset with an invariant TCRα chain, Vα24, exists, although in much lower quantities (4, 12–15). Because the CD1-iNKT cell system is highly conserved throughout evolution, and given the potent immune-deviating capacities of activated iNKT cells, this universal recognition system represents a potential therapeutic target for the treatment of autoimmune diseases (16, 17). Therefore, α-GalCer was tested in several autoimmune disease models such as diabetes and multiple sclerosis and found to induce regulatory or immune-deviating activity (18–21). Recently, the C-glycoside analog of α-GalCer (α-C-GalCer), a synthetic analog of α-GalCer, was reported to induce an enhanced production of IFN-γ, but reduced secretion of IL-4, thereby circumventing drawbacks of simultaneous production of both Th1 and Th2 cytokines. Thus, α-C-GalCer administration in certain tumor or infectious models whose protection relies on Th1 responses was found to result in a substantial improvement compared with α-GalCer itself (22).

1 Abbreviations used in this paper: iNKT, invariant NKT; α-GalCer, α-galactosylceramide; α-C-GalCer, C-glycoside analog of α-GalCer; CBA, cytometric bead array; CIA, collagen-induced arthritis; CII, collagen type II; m, murine.

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Collagen-induced arthritis (CIA) is an animal model of rheumatoid arthritis that has been used extensively to investigate disease pathogenesis and to validate novel therapeutic targets (23). A number of studies have provided some insight concerning the role of iNKT cells in this model of autoimmune joint inflammation. Therapeutic administration of OCH, an analog of α-GalCer shown to predominantly induce Th2-type cytokines, ameliorated arthritic disease in C57BL/6 and SJL mice, both strains that are normally less susceptible to CIA than DBA/1 mice. This protective effect was found to be due to IL-4 and IL-10 production and proved to be iNKT cell dependent (24). In another study, reduced incidence and arthritis scores were reported following CIA induction in Jo18-/- and CD1d-/- mice, leading the authors to suggest that iNKT cells were important effector cells in this model (25). Similar conclusions were drawn from work in which anti-murine (m)CD1 mAb treatment ameliorated CIA, and iNKT cell-deficient mice also proved more resistant against CIA and Ab-induced arthritis (26).

Finally, a recent report suggested that activation of iNKT cells in this model of autoimmune joint inflammation. Therapeutic administration of α-GalCer induced protection against CIA in an IL-10-dependent manner (27).

Although the model has been considered as a Th1-driven disease by several investigators, the role of IFN-γ, however, has been controversial, as follows: whereas some groups regard this cytokine as disease promoting, others have shown a marked protective role of IFN-γ in CIA (28). Moreover, the importance of the kinetic course of endogenous IFN-γ production in CIA has been generally appreciated, and timing of modulation of these levels greatly affects the outcome in terms of disease development and progression (29, 30).

Because of the ability of α-GalCer and the α-C-GalCer analog to induce production of large amounts of immunoregulatory cytokines with distinct Th1-Th2 profiles, we investigated whether a single early activation of iNKT cells by these glycolipids could alter the course of CIA in DBA/1 mice, with special emphasis on IFN-γ.

Our data demonstrate that a single administration of α-GalCer or α-C-GalCer during the development stage of CIA ameliorates disease severity, both clinically and histopathologically. We further show that this efficacy is hampered by endogenous IFN-γ production upon glycolipid administration and that different immunomodulatory mechanisms may be responsible for the protective effects of α-GalCer as opposed to the C-glycoside analog. Finally, these data indicate that clinical outcome of specific iNKT stimulation is highly time dependent, because glycolipid administration around time of onset was significantly less efficient.

Mice

Male, 9- to 10-wk-old DBA/1 mice were purchased from Janvier and housed following institutional guidelines. All animal experiments were approved by the Ethical Committee of Ghent University Hospital.

Induction and analysis of CIA

Bovine collagen type II (CII) (a gift from P. Sumariwalla, Kennedy Institute of Rheumatology, London, U.K.) was dissolved in 0.1 M acetic acid at a concentration of 4 mg/ml by stirring overnight at 4°C. Male DBA/1 mice were immunized intradermally at the base of the tail with 200 μg of CII emulsified in CFA (Difeo Laboratories) at the age of 8–12 wk. From day 21, mice were monitored daily for clinical symptoms of arthritis. Once arthritis was diagnosed, they were monitored daily for 14 days, after which they were euthanized. The clinical severity of arthritis was graded as follows: 3, pronounced edema and erythema in at least some digits; 2, slight edema or erythema involving the entire paw; 1, moderate edema and erythema involving the entire paw; 0.5, severe edema and erythema involving the entire paw; 0, normal paws. Clinical arthritis scores were reported following CIA induction in J11002, however, has been appreciated, and timing of modulation of these levels greatly affects the outcome in terms of disease development and progression (29, 30).

Histological evaluation

Knees were dissected postmortem, fixed in 10% formalized saline, decalcified, dehydrated, and embedded in paraffin. Sections of 7 μm were made and stained with H&E or with safranin O. Serial sections were scored on coded slides by two investigators. Inflammation was scored on a scale of 0 (no inflammation) to 3 (severe inflamed joint) depending on the number of inflammatory cells in the synovial cavity (exudate) and synovial tissue (infiltrate). Exudate and inflammatory infiltrate were both assigned individual scores. Loss of proteoglycans was scored on a scale of 0–3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage. Cartilage destruction was scored on a scale of 0–3, ranging from the appearance of dead chondrocytes (empty lacunae) to complete loss of the articular cartilage. Loss of bone was scored on a scale of 0–5 ranging from no damage to complete loss of the bone structure (33).

Ab treatment and anti-IFN-γ bioassay

To neutralize endogenous IFN-γ, 250 μg of anti-IFN-γ mAb (clone F3, rat IgG2a) was administered i.p. 24 h before (day 4 or 19 postimmunization) and 72 h after (day 8 or 23) α-GalCer administration. Bioactive IFN-γ was determined by titration on L929 cells using a cytopathogenic inhibition assay with mengovirus as a challenge (34). One unit/ml IFN-γ is the quantity necessary to exert a protective effect of 50% in L929 cells upon infection.

Cell lines

The B cell lymphoma A20 was originally obtained from the American Type Culture Collection, and its mCD1d-transfected counterpart A20CD1 was described previously (35, 36). DN3A4-1-2 hybridoma cells are derived from C57BL/6 NK1.1+ thymocytes, express the Vα14Jα18 TCR rearrangement, and show strong reactivity toward CD1d glycolipid Ags on the A20CD1 cell line (37). Cell lines were maintained and cultured in RPMI 1640 (Invitrogen Life Technologies), supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, 2 mM sodium pyruvate, and 5 × 10⁻³ M 2-ME (all from Invitrogen Life Technologies). This medium will be further referred to as complete RPMI 1640 medium.

In vitro analysis of NKT cell responses

Analysis of NKT cell hybridoma responses upon presentation of glycolipid ligands was performed, as described previously (8). Suspensions of A20 or A20CD1 cells were pulsed for 2–3 h at 37°C with 100 ng/ml glycolipids or vehicle and extensively washed before being seeded at 2 × 10⁵ cells/well in 96-well plates (Nunc). Responder NKT cell hybridomas were cultured at 5 × 10⁵ cells/well in the presence of pulsed A20 or A20CD1 cells. Supernatants were harvested after 16 h, and IL-2 levels were assessed by ELISA.

Preparation of cell suspensions and measurement of CII-specific proliferative T cell responses

For ex vivo analysis of T cell-proliferative capacity upon Ag-specific CII restimulation, draining inguinal and popliteal lymph nodes were removed from each group of mice 43 days after primary immunization and washed.
in complete RPMI 1640. Cells from two mice were pooled, and single-cell suspensions were prepared in complete RPMI 1640 and washed three times. Cells were counted in trypan blue to exclude dead cells. Lymphocytes (2 × 10^6/well) were cultured in 96-well plates with or without 40 μg/ml CII for 72 h. Sixteen hours before the termination of the cultures, 1 μCi of [3H]thymidine was added to each well. Cells were harvested onto glass fiber filters, and thymidine incorporation was determined using a Top Count liquid scintillation counter, according to the manufacturer’s instructions.

Cytokine detection and determination of serum IgG1/IgG2a ratios

On day 14 after disease onset, mice were i.p. injected with 10 μg of anti-CD3 mAb, followed by serum cytokine analysis at 1.5 h after anti-CD3 stimulation using cytometric bead array (CBA; BD Biosciences). Analogously, serum cytokine levels were determined in naive vs CII/CFA-immunized DBA/1 mice, after 2 and 16 h upon injection with α-GalCer or α-GalCer-IL-10, IL-2, IL-4, IL-5, IFN-γ, and TNF were detected using mouse Th1/Th2 cytokine CBA kit, and for IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 detection, mouse inflammation CBA kit was used. Tests were performed, according to the manufacturer’s instructions.

CII-specific IgG subtype ELISA kits were purchased from Chondrex to determine the IgG1 and IgG2a levels in serum derived from the different treatment groups, on day 14 after onset of arthritis. For the detection of IL-10, a commercially available ELISA was used (Immunosource). The ELISA was performed according to the manufacturer’s instructions.

Flow cytometry

Spleens were removed and teased apart. Erythrocytes from spleens were lysed with 0.17 M NH₄Cl, and the remaining lymphocytes were washed three times with Dulbecco’s PBS (Invitrogen Life Technologies). Cells were counted with trypan blue to exclude dead cells. To avoid nonspecific binding, the FcγR was blocked by preincubation of cells with saturating amounts of anti-FcγRIII/IIA mAb. Cells were incubated with the fluorochrome-conjugated mAbs at 4°C for 45 min. Cells were analyzed for fluorescence with a FACScan flow cytometer (BD Immunocytometry Systems) using the CellQuest Pro software.

Quantitative RT-PCR cytokine analysis of synovial tissue

Total RNA was isolated from frozen synovial biopsies, preserved in RNA later (Ambion), with RNase-free DNase set; Qiagen), and stored at −80°C until analysis. cDNA from total RNA was synthesized. Briefly, 0.5 μg of oligo(dT) primers (Promega) was added to 1 μg of total RNA in 10 μl of diethyl pyrocarbonate water and incubated for 5 min at 55°C. The solution was then quickly transferred to ice, and cDNA was prepared with Superscript II reverse transcriptase (Invitrogen Life Technologies), according to the manufacturer’s guidelines. RNAsin RNase inhibitor (Promega) was added at 40 U/μl. Primers were designed with Beacon Designer software (Premier Biosoft). Quantitative RT-PCR was performed with the qPCR Master Mix for SYBR Green I (Eurogentec) and 300 nM/l sense and antisense primers. Specificity of the SYBR Green assays was confirmed by melting point analysis. Gene expression of the housekeeping gene RPL-13a was used for normalization. Primer sequences are: mIL-18 sense, 5'-CTACTTACCAACAAGGGAGGCAGCAGAGTTCGACCTCG-3' and mIL-18 antisense, 5'-CTGCTGGTCCGCTCACTGGTGACAGG-3'. mIL-6 sense, 5'-GGCTTATTTGTTGAGCCATATAGTTGTTGAACAACACTTTAAG-3' and mIL-6 antisense, 5'-AGTGTCGACCACACTTATTGTCAG-3' and mRPL-13a sense, 5'-CTCTGTCGCTGTCGACAGGTTGTTGTTGTTT-3' and mRPL-13a antisense, 5'-TGCTGAGTGCAGATGTGCTAGTGTCTGTTT-3'.

Statistical analysis

For histological and cytokine data, the Fisher’s exact test and Mann-Whitney U test were used, respectively. For analysis of clinical data, we evaluated the following summary measures of arthritis manifestations: frequency of arthritis, time to onset of arthritis, and the maximum score of arthritis. Generalized linear models were used to calculate odds ratios and means with accordant SEs after adjustment for potential interexperiment variability. Thus, displayed graphs represent pooled data of the various studied treatment schedules from five independent experiments. Differences between groups were calculated by defining different contrasts. No adjustments were made for multiple comparisons (38). Value of p < 0.05 was considered significant. Graphpad Prism and SPSS software were used for all statistical data analysis.

Results

Therapeutic efficacy of early iNKT stimulation in CIA with α-GalCer

We initiated this study by assessing the effect of a single administration of α-GalCer in the preclinical phase on disease induction in CIA. Therefore, mice were immunized with a single intradermal injection of CII in CFA to induce CIA. At day 5 postimmunization, 2 μg of α-GalCer was administered i.p. Serum samples were taken at both 2 and 16 h following α-GalCer administration, to analyze IFN-γ and IL-4 production, and to confirm adequate iNKT cell activation (data not shown). Disease onset and progression were monitored daily in all mice by clinical scoring. However, once the animals developed arthritic symptoms, the course of arthritis was significantly attenuated in α-GalCer-treated animals vs controls (Fig. 1a). Maximum arthritis scores were also substantially reduced in treated animals (Table I). After 2 wk of arthritic disease, synovial biopsies and spleen samples were collected. The effector cytokines IL-6 and IL-1β, both important mediators of inflammation and tissue destruction in this model, were down-regulated in synovial tissue from α-GalCer-treated animals, as measured by quantitative real-time PCR analysis (Fig. 1b). In addition, we determined the levels of two key transcription factors that are indicative for the Th1/Th2 differentiation status of the Th cell repertoire in the spleens of treated mice. As indicated in Fig. 1c, levels of T-bet were significantly lowered, whereas GATA-3 levels were unaltered, suggesting a Th2 bias. Finally, Ag-specific proliferative responses after restimulation with CII in draining lymph node cells derived from α-GalCer-treated animals were found to be reduced, as compared with diseased PBS controls (Fig. 1d). Thus, disease amelioration was associated with decreased Ag-specific T cell reactivity toward CII.

Early α-GalCer administration protects against histopathological changes of mouse joints in CIA

After 2 wk of clinical arthritis, mice were sacrificed and knee joints were histologically examined to determine whether the amelioration of clinical disease activity correlated with improved histopathological parameters. Analysis of sections derived from vehicle-treated animals revealed that 87.5% of the joints assessed exhibited at least some evidence of pathology, most frequently inflammatory symptoms, graded as influx of inflammatory cells in synovium (infiltrate) and joint cavity (exudate). Consequently, the majority of joints showed evidence of depletion of proteoglycans, destruction of articular cartilage, and bone erosion often leading to loss of bone architecture (Fig. 2, c and d). In sharp contrast to the control group, 42.9% of joints from mice treated with α-GalCer demonstrated normal histopathological features (p = 0.008; data not shown), a condition that is representative depicted in Fig. 2, e and f. We did not observe any significant differences with regard to the mean parameter scores as follows: exudate mass (1.60; PBS vs 1.47; α-GalCer), inflammatory infiltrate (2.08; PBS vs 1.77; α-GalCer), bone erosion (1.97; PBS vs 1.92; α-GalCer), proteoglycan depletion (2.20; PBS vs 1.53; α-GalCer), and cartilage destruction (1.40; PBS vs 1.18; α-GalCer). However, we found a general shift from predominantly mild to moderately affected joints in the control group toward normal joint scores in the α-GalCer-treated cohort, for all parameters evaluated. No significant difference was present regarding percentages of severely affected joints, illustrating the incapacity of α-GalCer to confer protection to joints prone to development of severe arthritic symptoms. An overall overview of histopathological data is provided by the percentages of all joints assigned to be normal for the various parameters (Fig. 2e). Thus, the histopathological data indicate that a single administration of α-GalCer conferred to a
marked, albeit not complete, protection to develop synovial inflammation and cartilage and bone destruction.

In vivo neutralization of endogenous, α-GalCer-induced IFN-γ partially enhances protection against CIA in the early induction phase
Following α-GalCer treatment, high levels of IFN-γ accumulate in serum, with peak concentrations ~16 h after injection of the glycolipid. To address whether the endogenous IFN-γ production induced following α-GalCer injection accounts for the protective effect in CIA, we neutralized the endogenous IFN-γ production using neutralizing anti-IFN-γ mAb administration. A total of 250 μg of anti-IFN-γ mAb was administered i.p. 1 day before and 3 days following α-GalCer administration on day 5 postimmunization. Anti-IFN-γ treatment, without α-Galcer, was found to be associated with a significant reduction in the severity of arthritis (Fig. 3a). Of interest, mice that received accompanying IFN-γ neutralization together with α-GalCer treatment showed a slight additional benefit on disease progression (Fig. 3a). This supplementary ameliorative effect is represented by lower maximal arthritis clinical scores, as compared with mice treated with α-GalCer or anti-IFN-γ alone (Table I). Effectiveness of in vivo neutralization was confirmed by mouse IFN-γ viral inhibition assay (Fig. 3b).

Therapeutic efficacy of early iNKT stimulation in CIA with α-C-GalCer
Given the apparent importance of IFN-γ in α-GalCer-mediated protection in CIA and the debatable role of the cytokine in

<p>| Table I. Overview of pooled clinical data derived from five independent experiments |
|-------------------------------------------------|-----|-----------------|----------|-----------------|-----|</p>
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<th>Treatment Group</th>
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<th>p Value (compared to PBS)</th>
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<th>p Value (compared to PBS)</th>
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this model, we questioned whether early administration of α-C-GalCer, a synthetic analog of α-GalCer that induces enhanced production of IFN-γ, but reduced secretion of IL-4, could alter the course of disease. We first evaluated the potency of α-C-GalCer with regard to specific activation of iNKT cells, as compared with α-GalCer (Fig. 4). Therefore, both glycolipids were loaded on untransfected A20 B lymphoma cells and on A20 cells transfected with mCD1 (A20CD1). Upon Ag presentation of glycolipid ligand by CD1d on A20CD1 cells, the iNKT cell responder cells (DN3A4-1-2) become activated. The extent of activation can be quantified by measurement of IL-2 levels released in the supernatant. Of interest, considerably lower IL-2 levels were found upon stimulation with α-C-GalCer as compared with α-GalCer, indicating that the C-glycoside analog exhibits less potent stimulatory activity against iNKT cells.

Next, we chose to determine serum levels of IFN-γ, IL-2, IL12p70, TNF-α, IL-6, IL-4, IL-5, and IL-10 immediately after in vivo administration of α-GalCer, α-C-GalCer, or vehicle on day 5 in CII/CFA-immunized animals vs naive animals by CBA (Fig. 5), 2 and 16 h after glycolipid injection. No significant differences were found between levels induced by α-GalCer in naive vs CII/FIGURE 2. Histopathology of knee joints 2 wk postarthritis onset after prophylactic treatment with 2 μg of α-GalCer. Patellofemoral joint (a) and femorotibial joint (b) of an unimmunized, healthy B6 animal. c, Patellofemoral joint of control animal. Presence of exudate mass, inflammatory infiltrate, and limited bone erosion. Visible loss of matrix proteoglycans, indicated by destained cartilage layers, and some cartilage degradation. d, Similar conditions apply to femorotibial joints of these control animals. e, Patellofemoral joints of mice treated with α-GalCer. In most joints, inflammatory infiltrate was absent. Cartilage and bone were generally intact. f, Femorotibial joints of α-GalCer-treated animals. P, patella, T, tibia, F, femur, C, cartilage, JS, joint space, M, meniscus. g, A general shift toward normal joint phenotype was found after α-GalCer administration for all parameters examined. *, p < 0.01; **, p < 0.001; by Fisher’s exact test, compared with control.

FIGURE 3. α-Galcer-induced IFN-γ partially compromises clinical benefit of early α-Galcer administration in CIA. a, A total of 2 μg of α-GalCer was administered i.p. on day 5 postimmunization with CII in CFA, either with or without accompanying neutralizing anti-IFN-γ mAb treatment. Disease progression was monitored daily. p = 0.019; α-GalCer vs PBS, p = 0.017; α-GalCer + anti-IFN-γ vs PBS (mixed models, random slope). b, Endogenous IFN-γ, produced upon iNKT cell stimulation with α-GalCer, was effectively neutralized. Serum samples were taken 16 h after glycolipid administration and pooled per experiment and treatment group. Serum pools were analyzed by a mouse IFN-γ viral inhibition assay. One unit/ml IFN is the quantity necessary to produce a protective effect of 50% in L929 cells upon viral infection. n.d., Not detectable.

FIGURE 4. α-C-GalCer is a weaker iNKT cell agonist than α-GalCer. A20- and mCD1d-transfected A20CD1 B lymphoma cells were loaded with 100 ng/ml either α-C-GalCer or α-GalCer. Responder DN3A4-1-2 hybridoma iNKT cells were incubated for 16 h with either glycolipid- or vehicle-stimulated B lymphoma cell lines. Production of IL-2 was measured by standard sandwich ELISA. Only A20CD1 cells were capable of glycolipid presentation to the iNKT cell hybridoma responders (left panel).
CFA-immunized mice. In α-C-GalCer-treated mice, the overall levels of cytokines produced were lower than in α-GalCer-treated mice, with the exception of IL-12p70 and IL-6. The virtual absence of IL-2, TNF-α, IL-4, and IL-10 was particularly apparent. Whereas IFN-γ levels were found to be lower at 16 h after immunization in α-H9251-C-GalCer- vs α-H9251-GalCer-treated mice, additional kinetic experiments indicated that the higher levels of IL-12p70 induced by the C-glycoside analog at 16 h were accompanied by increased levels of IFN-γ at 24 h after administration, which exceeded the levels induced by α-GalCer itself (data not shown). In CII/CFA-immunized mice, the levels of IL-12p70 induced by α-C-GalCer were markedly higher compared with naive mice, perhaps reflecting an adjuvant effect. Immunized control animals that were not injected with α-GalCer were also included in the experiment, but no significant cytokine levels were found in the serum of these mice, except for IL-5 and IL-6.

We next evaluated the effect of the C-glycoside analog on clinical arthritis symptoms, in the presence or absence of neutralizing anti-IFN-γ Abs. Surprisingly, these experiments resulted in quite analogous findings as with α-GalCer treatment (Fig. 6a). Again, early administration of this glycolipid was found to confer protection against CIA progression and, similar to α-GalCer-mediated amelioration, anti-IFN-γ mAb treatment provided a slight additional benefit, as suggested by consistently lowered maximum scores, compared with mice treated with α-C-GalCer or anti-IFN-γ alone (Table I). IFN-γ neutralization was also confirmed by bioassay (data not shown).

To evaluate the impact of early iNKT cell activation, we determined the serum levels of anti-CII IgG1 and IgG2a in α-GalCer-, α-C-GalCer-treated animals vs PBS controls. We observed that in the α-GalCer treatment group significant higher levels of anti-CII Abs of the IgG1 subclass were observed 14 days after the onset of disease vs α-C-GalCer-treated mice or PBS controls (Fig. 6b), which is compatible with a Th2 immune deviation. The anti-CII IgG2a levels, however, were not significantly different between the treatment groups. This observation may indicate that the comparable clinical efficacy in CIA of both glycolipids is caused by distinct underlying mechanisms.

Clinical benefit of iNKT activation in CIA is time dependent

We next questioned whether the observed ameliorative effect of iNKT activation in CIA depends on time of glycolipid administration. Therefore, we also administered a single dose of 2 μg of α-GalCer and α-C-GalCer later during disease development, at day 20 postimmunization. Interestingly, no significant differences were observed in the frequency of arthritis (Table I). Maximum scores of treated mice were not significantly different from PBS-treated mice. Only the onset of arthritis seemed to be delayed in the glycolipid-treated mice compared with PBS-treated mice, especially in the α-GalCer group (α-GalCer + 3.95 days, p = 0.009; α-C-GalCer + 2.02 days, p = NS). Interestingly, in vivo neutralization studies using anti-IFN-γ mAb abolished this glycolipid-induced delay in disease onset (−4.68 days, p = 0.037) compared

![FIGURE 5. Serum cytokine levels in naive vs CII/CFA-immunized DBA/1 mice upon injection with PBS, α-GalCer, or α-C-GalCer. CII/CFA-immunized DBA/1 mice were injected with either PBS or 2 μg of α-GalCer or α-C-GalCer on day 5 postimmunization. As a control, naive DBA/1 mice were injected with either 2 μg of α-GalCer or 2 μg of α-C-GalCer. Cytokines were analyzed at 2 h (IL-2, TNF-α, IL-4, IL-10) and 16 h (IFN-γ, IL12p70, IL-5, and IL-6) after glycolipid injection using CBA. Bars, means (pg/ml) ± SEM. No cytokines were detected in sera from naive mice, before glycolipid administration.](http://www.jimmunol.org/2002.065.09.1209/)
with placebo, suggesting that IFN-γ has disease-attenuating properties late in the disease as opposed to its disease-promoting role in the early phase of CIA.

Glycolipid treatment in CIA does not induce long-term phenotypic alterations in lymphocyte and dendritic cell subsets

To determine whether early glycolipid treatment induced changes in lymphocyte subpopulations or in the frequency and activation status of APCs, which could account for the observed clinical efficacy, we performed an extensive phenotypical analysis. Initially, we characterized and quantified leukocyte subpopulations in immunized mice that received α-GalCer or α-C-GalCer treatment on day 5 vs controls, by flow cytometry on cells from draining lymph nodes on day 10 postprimary CIA immunization. We did not observe quantitative differences in CD11chiCD8α+ cells, CD11c-CD8α-, CD11c-CD206- (plasmacytoid) DC subsets, CD4+ and CD8+ T cell ratios, CD4+CD25+Foxp3+ regulatory T cells, F4/80+CD206+ (classical), and F4/80+CD206+ (alternatively activated) macrophages. The activation status, as investigated by CD69, CD40, CD40L, CD80, and CD86 expression, was also unaffected on the respective cellular subsets. Activated, memory CD44hi, CD4+, and CD8+ T cell numbers were also unaffected, indicating that α-GalCer did not induce differences in these conventional T cell subsets, as compared with controls. Furthermore, analysis of the MHCIihi, CD19+ B cell population did not reveal any differences.

Likewise, we analyzed lymph nodes and spleens derived from animals 14 days after onset of arthritis for the same panel of markers, but we could not discover any alterations. We also stained iNKT cells with α-GalCer-loaded CD1d tetramers and TCRβ, but we were unable to demonstrate significant changes in iNKT numbers among PBS, α-GalCer, and α-C-GalCer either in the presence or absence of anti-IFN-γ. Additionally, we were unable to demonstrate long-term changes in MHCIihi, CD11c+ dendritic cell numbers and CD40, CD80, and CD86 levels and frequencies of CD4+ and CD8α+ cells, and memory CD44hi T cells in mice treated with either glycolipid on day 20. These findings indicated that glycolipid treatment with α-GalCer or α-C-GalCer induced neither significant long-term changes in the frequency of conventional T cell subsets, nor in the number or activation status of APCs, particularly B cells and dendritic cells.

T cell cytokine production profiles after glycolipid administration suggest ligand-specific protective mechanisms

To examine whether T cell cytokine production was modulated in the glycolipid-treated animals, in the absence of apparent phenotypical changes in splenocyte or lymph node subsets, we assayed cytokine production upon TCR cross-linking. Therefore, we evaluated the levels of Th1-Th2 cytokines produced in vivo following polyclonal T cell activation by anti-CD3 mAb administration. We focused this analysis to mice treated in the early phase of CIA with
α-GalCer or α-C-GalCer because of the profound clinical benefit of either glycolipid at this time point. We polyclonally stimulated lymphocytes in vivo by injection of activating anti-CD3 mAb into glycolipid-treated mice on day 14 after onset of arthritis (Fig. 7a). After 1.5 h, IFN-γ, IL-2, IL-12p70, IL-4, IL-5, and IL-10 serum levels were quantified by CBA. Most notably, IL-10 was up-regulated in α-GalCer-treated mice as compared with the vehicle- and α-C-GalCer-treated cohorts. Surprisingly, the overall cytokine levels produced in α-C-GalCer-treated mice in vivo upon TCR cross-linking were found to be much lower, with the exception of IL-2. Interestingly, the suppressed cytokine levels in mice treated with the C-glycoside analog were restored in mice that received concomitant treatment with an anti-IFN-γ-neutralizing Ab (data not shown). Comparable results were obtained in serum 6 h after injection of anti-CD3 mAb (data not shown).

To further characterize the IL-10 up-regulation after α-GalCer administration during CIA development, we determined the IL-10 serum levels, 10 days after CII/CFA immunization, upon in vivo anti-CD3 polyclonal activation. Notably, only mice that were administered α-GalCer on day 5 showed up-regulated serum levels of IL-10, as compared with PBS- or α-C-GalCer-treated animals (Fig. 7b). Moreover, we demonstrate that this elevation is iNKT cell dependent, because we observed no alterations in Jα18^-/- mice. Of interest, IL-10 levels measured upon anti-CD3 stimulation in vivo were found to be comparable between PBS controls and Jα18^-/- mice, indicating that the produced IL-10 was derived from conventional T cells rather than from iNKT cells. Altogether, we conclude that a single administration of α-GalCer, but not α-C-GalCer, early after primary immunization results in early NKT cell-mediated bias toward IL-10 production.

Discussion

The advent of a specific iNKT-activating ligand, namely α-GalCer, has rapidly propagated the knowledge of this T cell subtype, and their unique immunomodulatory properties are now widely appreciated. Strong evidence has been gained on the physiologic importance of iNKT cells in human autoimmune diseases, suggesting their possible relevance in development of future therapies. The recent discovery of a number of self or bacteria-derived glycolipid ligands provides further clues to the precise immunological role of iNKT cells (39–44). During recent years, much interest has been drawn to the application of α-GalCer and its analogues for immune intervention in animal models of autoimmunity (45). Nevertheless, despite numerous reports on the attenuating properties of iNKT cell activation in these disease models, the underlying mechanisms often remain elusive.

Previous studies on the role of iNKT cells in CIA have often yielded conflicting results as follows: whereas studies using glycolipid-induced activation of iNKT cells suggested a regulatory potential in CIA, others proposed that these cells are important effectors (24–27). Repeated administration of the sphingosine-truncated analog of α-GalCer, OCH, was shown to be capable of conferring protective immune deviation in various animal models of autoimmunity, by means of inducing a Th2 bias (46–48). This concept also applied to CIA, in which IL-4 and IL-10 were found to be critical mediators of therapeutic benefit (24). Surprisingly, repeated therapeutic administration of α-GalCer, which was reported to induce Th2 immune deviation by several groups, did not elicit a clear improvement of arthritic disease (49, 50). By contrast, a recent study described beneficial effects of various prophylactic and (semi)therapeutic treatments with α-GalCer in CIA, and suggested that IL-10 would be an important determinant of the observed efficacy (27). Because one of the main differences between OCH and α-GalCer is the ability to induce IFN-γ, we evaluated this more in depth. Our goal was therefore to analyze the effect of a single early, prophylactic activation of iNKT cells by α-GalCer and articular disease progression with focus on IFN-γ. We describe several novel and unexpected features of iNKT cell stimulation in CIA.

First, we demonstrate that in mice injected with a single dose of 2 μg of α-GalCer on day 5 postimmunization with CII in CFA, the course of disease was significantly attenuated and associated with a Th2 immune polarization. Interestingly, disease symptoms usually appear ~day 20 after immunization, so the effect conferred by α-GalCer should be profound and lasting to influence the course of arthritis, 2 wk later. This clinical finding was confirmed by histopathology, and lower levels of IL-1β and IL-6, two principal cytokines responsible for inflammation and tissue destruction in CIA, were found in synovial tissue upon α-GalCer treatment. T-bet, considered a key transcription factor in development of a Th1 cell phenotype, was significantly down-regulated in spleens of α-GalCer-treated mice, whereas GATA-3 levels, which is its Th2-type counterpart, remained unaltered (51). This changed balance, observed in mice at day 14 postarthritus onset, indicates an altered Th1/Th2 balance following treatment with α-GalCer, in favor of a Th2-type immune response.

Secondly, our results indicate that the effect of iNKT cell activation in CIA is time dependent. Hence, semitherapeutic administration of glycolipid iNKT Ags was far less efficient in ameliorating arthritic disease progression and resulted in a delayed onset rather than reduced disease severity. The observation that the clinical efficacy of iNKT activation in CIA is time dependent puts emphasis on the dynamics of the iNKT cell’s influence on CIA development and progression. More generally, the profound impact of early intervention, in contrast with the lower responsiveness to our semitherapeutic protocol, indicates the essential importance of optimizing the timing of therapeutic iNKT activation in experimental autoimmune disease.

Thirdly, we unmask a biphasic role of IFN-γ released by iNKT cell activation in CIA. The role of IFN-γ in CIA has since long been a subject of debate. Early attempts to identify its role by the administration of IFN-γ or neutralizing anti-IFN-γ mAb yielded conflicting results, probably because of variations in timing, sites, and means of administration (29, 52–55). In two studies, anti-IFN-γ Ab treatment caused an increase in the number of arthritic limbs or severity of arthritis (55, 56), whereas in another study, the effect of anti-IFN-γ depends on the time of administration, early treatment being associated with reduced severity and late treatment being associated with aggravation of the disease (29). CIA development in IFN-γ^-/- or IFN-γR^-/- mice on DBA/1 background occurs faster and is more severe (56, 57). This points toward a role of IFN-γ during early development of the immune response that ultimately leads to onset of arthritis, but also suggests a strong influence on the actual course of disease afterward. Because IFN-γ is produced in substantial amounts after iNKT cell stimulation, we assessed the importance of IFN-γ in early α-GalCer-mediated protection. In vivo neutralization of functional IFN-γ, produced by iNKT cell stimulation, resulted in enhancement of clinical benefit compared with α-GalCer or anti-IFN-γ alone, thereby suggesting at least a partially compromising role for this cytokine regarding clinical benefit. By contrast, an opposite role of IFN-γ released by iNKT cells was delineated later in the disease in which IFN-γ was found to exert an important regulatory role by modulating the onset of the disease, rather than the severity of the joint inflammation. Thus, CIA appears to be a model in which, depending on the experimental conditions, either the autoimmune disease-limiting or the disease-aggravating effects of IFN-γ could predominate and influence the therapeutic benefit of iNKT cell activation.
Fourth, we demonstrate that the Th1 immune-deviating α-GalCer analog, α-C-GalCer, also confers protection in the early phase of CIA. We focused our attention on α-C-GalCer using the same treatment protocol because of its ability to induce a prolonged production of IFN-γ and IL-12, but decreased IL-4 production, by specific activation of iNKT cells (22). It was successfully applied to treat mouse models of malaria and melanoma metastases, suggested to be due to its predominant Th1 bias of iNKT cells. We confirmed and extended these observations by showing that α-C-GalCer induces a Th1 bias in naive animals by assaying an array of cytokines. Interestingly, the levels of IL-12 induced in CII/CFA immunized mice were three times higher than in naive DBA-1 mice, suggesting a potent influence of the adjuvant properties of CFA on the cytokine levels produced by iNKT cell stimulation. Surprisingly, clinical data obtained after early α-C-GalCer administration on day 5 were largely comparable to the results obtained with α-GalCer. The progression of CIA was also attenuated, and neutralizing anti-IFN-γ treatment resulted in an additional improvement. These data may further strengthen the hypothesis that therapeutic potential of iNKT cell-stimulating glycolipids is profoundly reliant on the induced cytokine pattern.

Finally, we propose two distinct mechanisms of α-GalCer vs α-C-GalCer administration that confer protection in the early induction phase of CIA based on the distinct Th1-Th2 cytokine levels induced upon polyclonal T cell activation in vivo. IL-10 produced by iNKT cells has been reported to induce regulatory dendritic cells, which in turn generate CD4+ regulatory T cells known to suppress immune responses (58). We found that the levels of IL-10 released in serum hours after α-GalCer administration were low. By contrast, a single dose of α-C-GalCer induced profound long-term functional changes, particularly with regard to IL-10, upon TCR cross-linking. Thus, IL-10 levels were markedly up-regulated upon anti-CD3 stimulation in vivo 14 days after the onset of arthritis, as compared with control animals. Consistent with this functional Th2 polarization, the levels of the transcription factor T-bet, crucial in Th1-mediated immune responses, were significantly reduced in spleens after early α-GalCer administration. It is conceivable that only minor levels of IL-10 produced upon injection of α-GalCer by the iNKT cells themselves may nevertheless exhibit potent immunomodulatory action on dendritic cells and/or certain lymphocyte subsets, although the details of the protective effect remain to be determined. These regulatory cell types may be responsible for higher, more relevant IL-10 production in a later stage of disease development and may account for more long-term immune deviation. It should be noted that the previously reported regulatory effects of IL-10 mediated by α-GalCer on dendritic cell function were primarily observed in repeated administration schedules. By contrast, our results clearly indicate that even a single administration of α-GalCer may provoke long-term enhanced capacity of IL-10 production upon TCR cross-linking in CIA. In addition to IL-10, we have tested serum levels of an array of pro- and anti-inflammatory cytokines both early and late after anti-CD3 injection, but no other significant differences from control animals were observed. Surprisingly, IL-4 levels were moderately reduced in glycolipid-treated animals upon polyclonal anti-CD3 activation. This may reflect the prominent role of overall cytokine balance rather than decreased levels of a particular cytokine on CIA development and progression (30, 50).

By contrast, α-C-GalCer seems to provoke another type of immune modulation. In comparison with α-GalCer, we demonstrate lower levels of cytokine production, immediately after injection of this glycolipid, most likely reflecting altered affinity of the CD1d-glycolipid complexes for the canonical TCR of iNKT cells. We confirmed this hypothesis by showing that iNKT cell hybridomas are less potently activated in vitro by α-C-GalCer as compared with α-GalCer. Interestingly, however, α-C-GalCer also induced long-term effects, as evidenced by cytokine levels measured by polyclonal anti-CD3 stimulation in vivo on day 14 after onset of arthritis. Rather than exhibiting features characteristic of a typical Th2 immune deviation, as observed with α-GalCer, treatment with α-C-GalCer resulted in a much lower production of the analyzed cytokines, reminiscent of a general T cell hyporesponsive state. Long-term hyporesponsiveness of iNKT cells and their apparent inability of cytokine production upon reactivation with α-GalCer have been established by several groups (59, 60), but the effect of other CD1d ligands has not been evaluated yet. In addition, an early iNKT cell-mediated bias toward an IL-10 production profile upon anti-CD3 stimulation was specific to α-GalCer, suggesting alternative modes of action as compared with α-C-GalCer. Our hypothesis that α-C-GalCer confers protection to CIA in a markedly distinct manner was further strengthened by the finding that only α-GalCer treatment modules CIH-specific IgG1 levels, which are indicative of Th2 immunity. Further studies are underway to unravel the mechanism(s) behind these findings.

In conclusion, our results provide further evidence for a crucial role of iNKT cells in CIA. We demonstrate that a single early administration of glycolipid Ag evokes potent immunomodulatory events leading to long-term therapeutic effects on arthritic disease progression. iNKT cell-derived IFN-γ at least partially compromises the observed clinical efficacy. Finally, the type of glycolipid iNKT ligand greatly delineates the outcome of iNKT stimulation, because α-GalCer most probably confers protection via a Th2 deviation of the immune response, most notably exemplified by increased IL-10 production, whereas α-C-GalCer is likely to induce its comparable clinical benefit by distinct pathways. The long-term nature of the observed effects may have profound implications for future therapies based on iNKT activation.

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Disclosures

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