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Brief Treatment with iNKT Cell Ligand α -Galactosylceramide Confers a Long-term Protection Against Lupus

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Abstract

CD1d presents glycolipid antigens such as α -galactosylceramide (α GalCer) to invariant natural killer T cells (iNKT). We have reported that activated iNKTs inhibit IL-10-producing autoreactive B cells, while promoting or leaving intact the normal B cell responses, making iNKT modulation an attractive therapeutic modality. Here, we report that a brief treatment of young lupus-prone (NZB/NZW)F1 (BWF1) mice with two injections of α GalCer conferred a long-term protection against lupus. Long-term repeated administrations of α GalCer, however, afforded no clinical benefit. These disparate clinical effects correlated with iNKT responsiveness. While a brief treatment with α GalCer enhanced iNKT responses upon in vitro recall, the long-term α GalCer treatment resulted in reduced iNKT responses in BWF1 mice. The improvement in disease with α GalCer treatment was associated with the reduced IL-10 production. Furthermore, iNKTs directly inhibited IL-10-secreting cells in vivo in reconstituted SCID mice and inhibited IL-10-secreting B cells in vitro in co-cultures. Thus, a brief treatment with a CD1d-binding glycolipid enhances iNKT responses, reduces IL-10 production, and delays the onset of lupus, whereas long-term repeated treatments induce marked iNKT hyporesponsiveness and do not affect disease outcome in BWF1 mice. Identifying glycolipid regimens that can modulate iNKT responsiveness will have important implications for developing iNKT-based therapies for autoimmune diseases.

Keywords

Rodent; T cells; autoimmunity; systemic lupus erythematosus

Introduction

Invariant natural killer T cell (iNKT) is a subset of T cells that recognize lipid antigens, such as α -galactosylceramide (α GalCer), in the context of the non-classical MHC class I molecule CD1d [1–3]. We have recently reported that iNKTs inhibit the production of autoantibodies that are the hallmark of autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis [4]. Consistent with the protective role of iNKT cells in autoantibody-mediated diseases, genetically lupus-susceptible (NZB $\times$ NZW)F1 (BWF1) mice rendered deficient in CD1d experience worse lupus nephritis than their wild-type littermates [5]. Similar results were reported using another model where autoantibody production is triggered by increased load of circulating apoptotic cells [6]. The absence or reduction of iNKTs as well as the absence of CD1d expression on B cells led to increased autoreactive B cell activation and symptoms of lupus-like disease, which was rescued by transferring iNKTs to deficient mice [6]. Intriguingly, while iNKTs reduce the production of rheumatoid factor and anti-DNA autoantibodies, these cells simultaneously increase total IgG levels and augment the production of antibodies against exogenous antigens [4]. iNKTs accomplish such precise, seemingly paradoxical, regulation of B cell subsets via CD1d that is expressed at a higher level on autoreactive B cells than on non-autoreactive B cells [4]. Such ability of iNKTs to differentially regulate normal versus autoreactive B cells makes them an ideal therapeutic target.

Compared to healthy subjects, patients with SLE and their relatives have reduced NKT cell numbers [7–9], suggesting that NKT cell impairment is a heritable trait. The reduced iNKT numbers in the relatives of patients with SLE correlate with the presence of autoimmunity [7]. Furthermore, whereas invariant V α 24J α Q (V α 24J α 18) T cell receptor (TCR) dominates double negative V α 24⁺ T cells at a high frequency in healthy subjects, this invariant TCR was reduced to undetectable levels in V α 24⁺ T cells from patients with active SLE [10]. V α 24J α 18⁺ T cells were restored to normal levels when patients were treated with corticosteroids [10]. Finally, whereas V α 24⁺V β 11⁺ double negative T cells from healthy subjects proliferated in the presence of α GalCer, five of the ten SLE patients tested exhibited no such response to α GalCer [8]. These data support a role of iNKT impairments in patients with SLE. In vivo treatments that can correct these NKT cell impairments might be of therapeutic value.

Here, we investigated the clinical effects, iNKT responses, and cytokine production in BWF1 mice treated with α GalCer. Results show that a brief treatment with α GalCer augments iNKT responses and confers long-term protection against lupus. A long-term repeated treatment with α GalCer, however, elicits selective iNKT hyporesponsiveness and affords no clinical benefit.

Materials and Methods

Mice

BALB/c SCID, NZB, and NZW mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). NZB and NZW mice were bred locally to generate BWF1 mice that develop T cell-dependent autoantibody-mediated lupus nephritis [11]. Animals used were bred and housed in animal colonies at the University of Cincinnati and UCLA. V α 14^{Tg} BALB/c mice that are transgenic for iNKT TCR [12] and J α 18^{-/-} BALB/c mice that have

no iNKTs [13] were provided by Dr. A. Bendelac and Dr. M. Taniguchi, respectively. Female mice were used in all experiments. Experiments were performed according to the approved institutional guidelines.

α GalCer Treatment

Synthetic α GalCer (KRN 7000) was kindly provided by Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan). Animals were treated twice a week i.p. with 4–6 μ g α GalCer or the same volume of control vehicle (0.025% polysorbate-20 in PBS).

Assessment of Lupus Disease

Proteinuria was measured on a 0–4+ scale using a colorimetric assay strip for albumin (Albustix, Bayer Co., Elkhart, IN), where 0=absent, 1+ 30 (mild), 2+=100 (moderate), 3+=300, and 4+ 2,000 mg/dl (severe). Severe proteinuria was defined as 300 mg/dl on two consecutive examinations, as described previously [14]. IgG anti-DNA Abs were measured by ELISA, as described [5, 14]. Anti-DNA Ab titers are expressed as units per milliliter (U/ml) using a reference-positive standard of pooled serum from 1-year-old BWF1 mice. Experiments shown in Fig. 1 versus Fig. 2 used different sets of standard serum. Hence, the results of anti-double-stranded DNA (dsDNA) antibodies are not comparable between Figs. 1 and 2.

Cytokine Assay

Spleen cells (10^6 cells per ml) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2×10^{-5} M 2-ME, 20 mM HEPES, 1 mM sodium pyruvate, and 100 μ g/ml gentamicin and various stimulants as described in the figure legends. Supernatants collected were assayed for cytokine levels by ELISA, as described [5].

Flow Cytometry Analysis

Freshly prepared or cultured spleen cells were incubated with anti-CD16/32 (2.4 G2, PharMingen) to block Fc γ RII/III, followed by staining with various conjugated mAbs (all PharMingen), as indicated in the figure legends. iNKTs were identified by CD1d/ α GalCer tetramer staining, as described [15]. Stained cells were analyzed using a Becton Dickinson FACSCalibur flow cytometer (Mountain View, CA, USA) and CellQuest software. Absolute cell numbers of each cell subset were calculated by the percentage of staining cells times total cell numbers.

Cytokine Secretion Assay

We detected IL-10-secreting cells using the MACS Cytokine Secretion Assay Kit (Miltenyi Biotec, Auburn, CA, USA), as described [4, 15, 16]. Briefly, spleen cells prepared from the reconstituted BALB/c SCID mice or cells from the mixed cultures of V α 14^{Tg} T cells plus J α 18^{-/-} B cells were incubated at 37°C for 45 min with the cytokine Catch Reagent that attaches to all leukocytes via CD45 antigen and binds to the specific cytokine. After washing, cells were stained with PE-conjugated IL-10 detection mAbs, counterstained with anti-CD11b mAb, and analyzed using FACSCalibur.

SCID Reconstitution Experiment

B cells were purified from the spleen of J α 18^{-/-} mice using anti-CD45R (B220) microbeads on an autoMACS magnetic cell separator (Miltenyi Biotec, Auburn, CA, USA). J α 18^{-/-} B cells were used to avoid any contaminating iNKTs. Ten million of these B cells (>97% pure) were injected i.v. into BALB/c SCID mice. The B cell-reconstituted SCID mice were then transferred i.v. with six million iNKTs (TCR β ⁺CD1d- α GalCer dimer⁺), which were enriched from the spleen of V α 14^{Tg} mice, as described [4]. The purity of iNKTs was >92%.

Control animals received the same numbers of B220⁺ cell-depleted spleen cells from J α 18^{-/-} mice. Animals received an i.p. injection each of 5 μ g LPS and 4–6 μ g α GalCer separately prior to reconstitution with cells. To verify the reconstitution, spleen cells harvested from these mice were analyzed for TCR β ⁺CD1d- α GalCer dimer⁺ cells 4 days after the transfer, as described previously [4]. As expected, the recipients of V α 14^{Tg} T cells had iNKTs, whereas recipients of J α 18^{-/-} T cells had no iNKTs. Spleen cells from the reconstituted mice were analyzed for IL-10-secreting cells using the above described cytokine secretion assay.

Statistics

The statistical significance was analyzed using Student's *t* test or Mann–Whitney *U* test. Differences were considered significant when *p* value was <0.05. Proportions of mice with proteinuria were compared using a log-rank test.

Results

A Brief Treatment with α GalCer Confers a Long-term Protection Against Lupus Nephritis

To determine the *in vivo* effects of iNKTs on spontaneously developing autoantibody-mediated disease, we treated BWF1 mice with α GalCer or vehicle and monitored them for clinical evidence of nephritis and serum IgG anti-dsDNA Ab. Remarkably, treatment of young BWF1 mice with only two α GalCer injections led to a prolonged reduction in renal disease and IgG anti-DNA Abs (Fig. 1). The 50% prevalence of severe proteinuria was delayed by 17 weeks in α GalCer-treated animals compared to vehicle-injected control animals. Thus, *in vivo* iNKT activation for a brief period can confer a long-term protective effect on lupus disease.

Long-term, Repeated Administration of α GalCer Has No Effect on Lupus Nephritis

We then determined the effect of long-term, repeated administrations of α GalCer on lupus disease. We found that long-term treatment in mice at a preclinical stage (Fig. 2a), in mice with early clinical disease (Fig. 2b), or in mice having full-blown disease (Fig. 2c) had no effect on disease progression, as indicated by proteinuria and serum IgG anti-dsDNA Ab. Thus, in contrast to the above protective effects of short-term α GalCer treatment, the long-term continuous treatment of BWF1 mice had no significant effect on lupus nephritis.

Brief α GalCer Exposure Boosts iNKT Responses, Whereas Long-term Repeated α GalCer Treatments Reduce iNKT Responsiveness in BWF1 Mice

To assess the mechanisms underlying the disparate clinical effects of short-term versus long-term α GalCer regimens, we first analyzed iNKT responses after single or multiple repeated injections of α GalCer in BWF1 mice. Young BWF1 mice were injected once with vehicle or α GalCer, and their spleen cells cultured with increasing concentrations of α GalCer. As expected from previous studies in normal animals, addition of α GalCer to cultures of spleen cells from control, vehicle-injected BWF1 mice elicited increases in IFN- γ , IL-13, and IL-4 levels and in spleen cell proliferation. The levels of these cytokines and spleen cell proliferation were significantly higher, however, in mice that were primed *in vivo* with α GalCer (Fig. 3). Thus, brief *in vivo* priming with a glycolipid antigen boosts iNKT responses in lupus-prone mice.

Next, we analyzed iNKT cell responses to long-term repeated treatments with α GalCer in BWF1 mice. Surprisingly, we found that administration of α GalCer twice a week for >10 weeks resulted in an almost complete iNKT cell unresponsiveness as compared to vehicle-injected control BWF1 mice (Fig. 4). This unresponsiveness is glycolipid antigen-specific, since Con A and anti-CD3 elicited similar cytokine responses in α GalCer- and vehicle-

injected mice (Fig. 4). Such iNKT cell unresponsiveness is not associated with attrition in iNKT cell numbers after long-term repeated exposures to a glycolipid antigen, as shown in Fig. 5.

α GalCer Treatment of Young BWF1 Mice Reduces IL-10 Production

To begin to investigate the mechanisms underlying α GalCer-mediated improvement in disease in BWF1 mice shown in Fig. 1, we measured cytokines, IL-2, IL-4, IL-10, IL-13, TGF β 1, and IFN- γ in spleen cell culture supernatants of BWF1 mice 6 months after treatment with two injections of α GalCer. IL-10 production was consistently lower in spleen cell cultures of α GalCer-treated than in vehicle-injected BWF1 mice (Fig. 6a), whereas other cytokines were increased or unchanged compared to vehicle-injected control mice (data not shown).

Reduced IL-10 levels in α GalCer-treated BWF1 mice could be due to reduced numbers of IL-10-producing cells owing to improved disease or due to a direct of iNKT cells on IL-10-producing cells. To test the possibility that iNKTs directly reduce IL-10-producing cells, we conducted adoptive transfer studies in SCID mice that were implanted with B cells (from Ja.18^{-/-} mice to avoid any contaminating iNKT cells). As shown in Fig. 6b, B cell-reconstituted SCID mice that were transferred with isolated iNKTs had lower numbers of IL-10-producing cells than SCID mice that received no iNKTs (i.e., Ja.18^{-/-} T cells). Thus, iNKTs can directly reduce IL-10-producing cells in vivo.

To further investigate if iNKTs inhibit IL-10 production by B cells, as reported recently [4], we co-cultured V α 14^{Tg} T cells and Ja.18^{-/-} B cells with LPS or CpG and enumerated IL-10-secreting B cells. V α 14^{Tg} T cells were used in these experiments as a rich source of iNKT cells as well as to obviate the possibility that iNKT cells might have undergone anergy or activation-induced cell death during their isolation using dimers in Fig. 6b. Results show that the addition of α GalCer to LPS or CpG-containing cultures resulted in 1.7–1.8-fold reduction in the proportion and numbers of IL-10-secreting B cells (Fig. 6c). Taken together, these data suggest that α GalCer-activated iNKTs inhibit IL-10-producing B cells.

Discussion

Ample evidence supports a protective role of iNKTs in inflammatory conditions [17–21]. We have reported several lines of evidence suggesting a regulatory role of CD1d system in lupus. First, iNKTs inhibit autoreactive B cells [4]. Second, deficiency of CD1d exacerbates lupus dermatitis [22], whereas α GalCer treatment ameliorates dermatitis in the MRL-lpr model [16]. Third, CD1d deficiency exacerbates lupus induced by hydrocarbon oil tetramethylpentadecane (TMPD) [15], whereas α GalCer treatment ameliorates TMPD-induced lupus in BALB/c mice [23]. Fourth, CD1d deficiency worsens lupus nephritis in BWF1 mice [5]. Consistently, aged Ja.18-deficient mice develop antibodies against double-stranded DNA and features of immune nephritis [24]. A suppressive role for iNKTs was also reported in a model where autoantibody production is triggered by an increased load of circulating apoptotic cells [6]. Contrary to these observations supporting a protective role of CD1d-reactive T cells in lupus, other reports showed that an anti-CD1d antibody prevents lupus [25], and α GalCer treatment worsens it in BWF1 mice [26, 27]. Analogously, treatment with an anti-CD1d antibody reduces collagen-induced arthritis [28], whereas iNKT activation protects against arthritis [19, 29]. Such paradoxical observations prompted us to further probe the role of iNKTs in lupus.

In this study, a brief treatment with α GalCer conferred a long-term protection against lupus in BWF1 mice. Long-term repeated administrations of α GalCer, however, had no significant effect on disease. Our data offer a potential mechanism to explain such disparate

effects of iNKT activation on lupus disease. We show that a brief exposure to α GalCer boosts iNKT response in BWF1 mice. Such transient restoration of iNKT responses appears to re-set the autoimmune process, conferring long-term protection against lupus. Upon a prolonged and repetitive exposure to the same glycolipid antigen (α GalCer), however, iNKTs undergo profound hyporesponsiveness, which correlates with a lack of therapeutic benefit to α GalCer in BWF1 mice. Further studies are needed to critically link iNKT cell responsiveness to clinical effects in lupus, especially as to when or after how many injections of α GalCer the iNKT cell hyporesponsiveness sets in.

The therapeutic benefit of short-term iNKT activation in conferring protection has been reported in animal models of collagen-induced arthritis, autoimmune encephalomyelitis (EAE), and bronchial asthma [19–21, 30]. In an animal model of asthma, a single subcutaneous injection of α GalCer at the time of intranasal antigen challenge abrogates disease, while continuous α GalCer administrations increase disease [20, 21]. In EAE, a single α GalCer injection prior to induction of disease prevents it, but enhances disease when injected at the time of disease induction [30]. In BWF1 mice, initiating α GalCer treatment at 20 weeks of age when mice already have disease modestly accelerated 50% prevalence of moderate (2+) proteinuria by ~7 weeks [26]. Thus, the timing of treatment in relation to the stage of disease and the route and frequency of administration of glycolipid antigens may all influence its therapeutic efficacy in inflammatory diseases [31].

In a previous study, whereas α GalCer treatment suppressed TMPD-induced renal disease in BALB/c mice, it increased renal disease in SJL mice [23]. Similarly, α GalCer can prevent EAE in B10.PL, PL/J, and C57BL/6 mice [30, 32, 33], but had little effect, or even exacerbated disease in a relapsing–remitting model of EAE in SJL mice [33]. Furthermore, iNKTs can modulate certain disease manifestations, but not others, in the same animal strain. For example, α GalCer treatment and CD1d deficiency modulate dermatitis, but not nephritis, in the MRL-lpr model [16, 22]. Delineating mechanisms underlying these divergent effects of iNKTs in different models of inflammatory diseases will help pave the way for iNKT-based therapies in humans.

It is unclear how iNKTs might regulate autoimmune diseases. We have recently reported that iNKTs inhibit CD1d^{hi} autoreactive B cells and reduce the number of IL-10-secreting B cells in vitro [4]. In this study, we show that IL-10 production by spleen cells was reduced in α GalCer-treated mice. The reduced IL-10 production is not likely owing to α GalCer-induced clinical effect, as iNKTs reduced IL-10-secreting cells in vivo in the reconstituted SCID mice and in vitro in co-cultures of purified iNKT and B cells. Our finding is likely important, because autoreactive B cells from patients with SLE produce increased amounts of IL-10 [34]. We have also recently shown that anti-DNA B cells spontaneously express higher levels of IL-10 than non-anti-DNA B cells [4]. In vivo neutralization of IL-10 reduces autoantibody production in reconstituted hu-SCID mice [34] and suppresses nephritis in BWF1 mice [35]. A pilot trial of an anti-IL-10 mAb also showed reduced disease activity in SLE patients [36]. Thus, the ability of iNKT cells to regulate IL-10-producing B cells might underlie their capacity to suppress lupus. In EAE, however, IL-10 plays a role in iNKT cell-mediated protection from autoimmune disease [33], suggesting that different mechanisms may underlie iNKT cell effects in different inflammatory diseases.

Given the limited polymorphic nature of CD1 genes with ensuing therapeutic advantage over highly polymorphic MHC class I and class II systems, there has been a tremendous interest in understanding iNKT biology in inflammatory diseases. Results of this study link the clinical effect of treatment with an iNKT ligand to iNKT responsiveness. Enhanced iNKT responsiveness after a brief treatment correlates with protection from disease, whereas

reduced iNKT responsiveness after a prolonged treatment correlates with a lack of therapeutic effect. We further suggest a mechanism whereby iNKTs might protect against lupus by suppressing IL-10 production. These data have important implications for the development of new avenues to prevent relapses in autoimmune diseases.

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Fig. 1.

A brief treatment with α GalCer confers long-term protection from lupus in BWF1 mice. Seven-week-old BWF1 mice were treated with α GalCer (4 μ g i.p.) or vehicle twice at a 3-day interval. **A** Results are shown as the percentage of mice with severe proteinuria (≥ 300 mg/dl) on two consecutive assessments. $**P$ 0.01 (log-rank test; $n=21$ mice per group pooled from two separate experiments). **B** The animals were bled and their sera tested for IgG anti-dsDNA Ab ($*P$ 0.05; mean $\pm$ SE; $n=12$ mice per group)

Fig. 2.

Long-term repeated administration of α GalCer does not protect from lupus in BWF1 mice. Five-week-old mice at a preclinical stage, i.e., without circulating anti-dsDNA Abs or proteinuria ($n=22$ mice per group; **a, d**); 18-week-old mice with early clinical disease, i.e., with IgG anti-dsDNA Abs but with mild or no proteinuria ($n=21$ mice per group; **b, e**); and 26-week-old mice with advanced disease, i.e., with IgG anti-DNA Abs and moderate proteinuria ($n=12$ mice per group; **c, f**) were treated with α GalCer or vehicle twice a week for 11–18 weeks. Results are shown as the percentages of mice with severe proteinuria (> 300 mg/dl) on two consecutive assessments (**a–c**) and as the mean $\pm$ SE units per milliliter serum IgG anti-dsDNA Ab (**d–f**)

Fig. 3.

Effect of short-term administration of α GalCer on iNKT cell responses in BWF1 mice. **a** Cytokine responses. Seven-week-old BWF1 mice were injected i.v. once with 4 μ g α GalCer or vehicle alone (five mice per group). Spleen cells from these mice were cultured without or with 10–100 ng/ml α GalCer for 2 days, and culture supernatants assayed for cytokines by ELISA. Results are shown as the mean $\pm$ SE nanogram per milliliter. * P 0.01; # P 0.05 compared to vehicle control mice. Similar data were obtained in another five each of 14-week-old BWF1 mice (data not shown). **b** Proliferative responses. Ten-week-old BWF1 mice were injected i.v. with 4 μ g α GalCer or vehicle. Spleens were harvested 2 h later, and spleen cells cultured with medium alone or 50 ng/ml α GalCer for 48 h, with 3 H thymidine in the last 16 h of culture. Results are shown as the mean $\pm$ SE ($n=3$ per group). * $P<0.01$ compared to vehicle-injected mice

Fig. 4. Effect of long-term administration of α GalCer on iNKT cell responses in BWF1 mice. Nine-week-old female BWF1 mice were treated with α GalCer (4 μ g i.p. twice a week) or control vehicle for 3 months. Three months later, mice were sacrificed and their spleen cells cultured with medium alone, α GalCer (50 ng/ml), Con A (5 μ g/ml), or plate-bound anti-CD3 (5 μ g/ml) for 2 days. Supernatants were assayed for cytokines, which are shown as the mean+SE. * P <0.05; n =4 mice per group

Fig. 5.

Effect of αGalCer treatment on iNKT cell numbers in BWF1 mice. Seven- to 9-week-old BWF1 mice were treated with αGalCer (4 μg i.p. twice a week) or equal volume of vehicle for a short-term (1 week, as described in Fig. 1a) (**a**) or for a long-term (12 weeks, as described in Fig. 2) (**b**). At 8–9 months of age, animals were euthanized and their spleen cells analyzed for CD1d/αGalCer tetramer⁺ TCRβ⁺ cells (*n*=6–9 mice per group). Splenic iNKT cell numbers were not statistically significantly different between αGalCer- and vehicle-treated animals.

Fig. 6.

Effect of αGalCer treatment on IL-10 production. **a** Spleen cells were harvested from BWF1 mice 6 months after treatment with αGalCer or vehicle (as in Fig. 1) and cultured with LPS without or with αGalCer. Supernatants were assayed for IL-10 by ELISA (* $P < 0.05$; $n = 10$ mice per group). **b** BALB/c SCID mice were reconstituted with B cells and T cells (CD1d-αGalCer dimer⁺ cells from Vα14^{Tg} mice or Jα18^{-/-} B cell-depleted spleen cells), as described in “Materials and Methods”. Spleen cells from the reconstituted mice were analyzed for IL-10-secreting cells using a cytokine secretion assay. Co-staining with CD11b was performed to distinguish between adoptively transferred cells (CD11b⁻) and SCID

mouse spleen cells (CD11b⁺). Numbers of IL-10-secreting cells per million spleen cells are shown. Results represent two independent experiments. **c** T cells from V α 14^{Tg} mice and B cells from J α 18^{-/-} mice were cultured overnight with LPS or CpG and vehicle or α GalCer. IL-10-producing B cells, as detected by cytokine secretion assay, are shown as percentages (%) and total cells per million in the culture. Results represent two independent experiments