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The natural killer T cell ligand α -galactosylceramide prevents or promotes pristane-induced lupus in mice

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Abstract

Systemic lupus erythematosus is a systemic autoimmune disease characterized by inflammation in organs such as kidneys and presence of autoantibodies against nuclear antigens. We have previously shown that CD1d deficiency in BALB/c mice exacerbates lupus nephritis and autoantibody production induced by the hydrocarbon oil pristane. Here, we have tested the impact of activating CD1d-restricted natural killer T (NKT) cells on pristane-induced lupus-like autoimmunity in BALB/c and SJL mice. Repeated *in vivo* treatment of pristane-injected BALB/c mice with the NKT cell ligand α -galactosylceramide (α -GalCer) prior to the onset of florid disease suppressed proteinuria, in a manner that was dependent on CD1d and IL-4 expression. In sharp contrast, however, similar treatment of pristane-injected SJL mice with α -GalCer resulted in increased proteinuria. Consistent with these dichotomous effects of NKT cell activation on the development of lupus-like autoimmunity, NKT cells in BALB/c and SJL/J mice exhibited a mixed Th1/Th2 and a Th1-biased cytokine production profile, respectively. These findings demonstrate that NKT cell activation with α -GalCer suppresses or promotes pristane-induced lupus-like autoimmunity in mice, in a strain-dependent manner.

Keywords

NKT cells; CD1d; Systemic lupus erythematosus; Glycolipids; Immunotherapy

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterized by antinuclear autoantibodies and multiorgan tissue injury, including immune complex glomerulonephritis [1]. The development of SLE is associated with the generation of autoreactive T helper (Th) cells and a decline in regulatory or suppressor T cells. However, the precise nature and specificity of regulatory T cells that can control the pathogenesis of SLE remain largely undefined.

NKT cells are a unique subset of T lymphocytes that express receptors for both T cells and NK cells [2–4]. Prototypical NKT cells express a semi-invariant TCR encoded by *V α 14-J α 18*/

Vβ8 gene segments in mice and homologous *Vα24-Jα18/Vβ11* gene segments in humans. NKT cells recognize glycolipid antigens presented by the MHC class I-like molecule CD1d and play an important role in regulating immune responses [2,3,5]. Mice and humans that are genetically susceptible to autoimmune diseases, including SLE, have reduced numbers of, and functional defects in, their NKT cells [2,3]. For example, C57BL/6-*lpr/lpr* and MRL-*lpr/lpr* mice, which are genetically susceptible to lupus-like autoimmunity, have defects in the numbers and functions of NKT cells [6,7]. Likewise, humans with SLE disease have reduced numbers of NKT cells [8–11], which selectively recover during remission [9].

Although several potential candidates have been suggested, the natural ligands of NKT cells remain unknown. Therefore, many investigators have studied the immunomodulatory properties of α -galactosylceramide (α -GalCer), a marine sponge-derived glycolipid that potently activates both mouse and human NKT cells [2–5]. Administration of α -GalCer to mice or humans results in the rapid production of Th1 and Th2 cytokines by NKT cells, followed by trans-activation of, and cytokine production by, a variety of innate and adaptive immune cells [2–5]. In most mouse strains, α -GalCer treatment promotes the generation of Th2 immune responses [12,13], which has been exploited for the development of immunotherapies for autoimmune diseases [14].

In contrast with most organ-specific autoimmune diseases, SLE cannot be easily characterized as a Th1- or Th2-dominant disease [1], and the precise pathology depends on the experimental model that is utilized. A number of genetic models, including MRL-*lpr/lpr* and (NZB/NZW) F1 mice, have been invaluable for studying the pathology of SLE. We have recently shown that α -GalCer alleviates inflammatory dermatitis, but not kidney disease, in MRL-*lpr/lpr* mice [7]. Additionally, it was recently shown that α -GalCer exacerbates lupus in old (NZB/NZW) F1 mice, which correlated with the induction of abnormal pathogenic Th1 immune responses [15]. In addition to these hereditary models, lupus-like autoimmunity can be induced in otherwise non-autoimmune-prone mice by injection of hydrocarbon oils such as pristane [16, 17]. Pristane-induced lupus mirrors many of the characteristics of human lupus, with regard to both clinical features and autoantibody profiles. We have recently shown that pristane injection results in a decline in NKT cell numbers and functions in BALB/c mice [18], and that CD1d deficiency exacerbates pristane-induced SLE-like disease in these animals [18], suggesting that NKT cells play a suppressive role in the pathogenesis of pristane-induced lupus.

Here, we have investigated the capacity of α -GalCer to modulate pristane-induced lupus in BALB/c and SJL mice. Surprisingly, we found that repeated α -GalCer treatment prevents pristane-induced lupus in BALB/c mice, but augments disease induced in SJL mice. These results suggest that NKT cells can play both suppressive and pathogenic roles in the regulation of lupus, in a manner that is genetically determined.

Results

NKT cell numbers and functions in BALB/c and SJL mice

Many mouse strains, especially SJL mice, are susceptible to pristane-induced lupus nephritis, and BALB/c mice are most commonly utilized in this model [17]. Therefore, we selected BALB/c and SJL mice for our studies. First, we compared NKT cell numbers in these animals with those in B6 mice, which are most commonly used for studies on NKT cells. Evaluation of NKT cell numbers with CD1d tetramers revealed similar prevalence of NKT cells in B6 and BALB/c mice (Fig. 1A). Consistent with prior studies [19,20], NKT cells in SJL mice were drastically reduced as compared with B6 and BALB/c mice.

Second, we examined serum IL-4 and IFN- γ levels at different time points after α -GalCer treatment *in vivo*. Consistent with prior studies [2,12,13], α -GalCer treatment of B6 mice

resulted in an early burst of IL-4 and slightly delayed IFN- γ production (Fig. 1B). In BALB/c mice, IL-4 production was slightly delayed, whereas IFN- γ production peaked earlier as compared with B6 mice. In SJL mice, IL-4 production was minimal, and IFN- γ production was reduced and peaked earlier, as compared with B6 mice. Third, we compared the capacity of repeated *in vivo* α -GalCer treatment (three injections, 6 μ g/injection) to modulate serum IgE levels that serve as a surrogate marker of Th2 responses. Results showed a robust increase in serum IgE levels in B6 and BALB/c mice, but no significant alterations in SJL mice (Fig. 1C).

Fourth, we measured *ex vivo* responses of splenocytes to α -GalCer activation. α -GalCer-stimulated splenocyte cultures from BALB/c mice produced similar amounts of IFN- γ and slightly enhanced levels of IL-4 as compared with splenocyte cultures from B6 mice (Fig. 1D). In sharp contrast, cultures from SJL mice produced little IL-4 but slightly enhanced levels of IFN- γ , suggesting that the remaining NKT cells in SJL mice have a Th1 bias. Fifth, to confirm and further extend the latter findings, we stimulated splenocytes *ex vivo* with α -GalCer or PMA plus ionomycin and measured intracellular cytokine levels of CD1d tetramer⁺ cells. Results indicated enhanced prevalence of IFN- γ -secreting NKT cells from SJL mice as compared with B6 and BALB/c mice (Fig. 1E).

Finally, we evaluated the effects of α -GalCer treatment on an ongoing adaptive immune response. We immunized mice with the protein antigen hen egg lysozyme (HEL) in adjuvant, treated animals with vehicle or α -GalCer on days 0, 4, and 7, and measured recall responses to HEL on day 11. Results showed that α -GalCer induced Th2 deviation in the anti-HEL response in B6 and BALB/c mice, but instead moderately promoted Th1 deviation in SJL mice (Fig. 1F, G). Collectively, our results suggest that NKT cell numbers and functions in B6 and BALB/c mice are similar, although not identical, that SJL mice have reduced numbers of NKT cells with a bias towards IFN- γ production, and that α -GalCer has a tendency for promoting Th1 rather than Th2 responses in SJL mice.

α -GalCer protects BALB/c mice against pristane-induced SLE

Next, we tested the capacity of α -GalCer to modulate the development of lupus in pristane-injected BALB/c mice. Mice were injected with a single dose of pristane for induction of lupus and treated with vehicle or α -GalCer (6 μ g/injection) twice a week for a period of 1 month, starting from the day of pristane administration. Every 3 months after pristane injection, urine and blood were collected for evaluation of proteinuria and lupus-associated antibodies, respectively.

Proteinuria data pooled from four independent experiments are shown in Fig. 2A and are summarized in Table 1. Proteinuria grades in α -GalCer-treated BALB/c mice were significantly reduced as compared with vehicle-treated mice. Strikingly, α -GalCer-treated mice were completely protected from the development of severe ($\geq 3+$) proteinuria, whereas 40% of mice in the control group developed severe proteinuria ($p=0.03$ at 9 months and $p<0.01$ at 12 months).

Levels of anti-double-stranded DNA (anti-dsDNA) antibodies were not significantly different at any individual time point, although there was a trend for reduced anti-dsDNA antibody titers in the serum of α -GalCer-treated mice as compared with vehicle-treated mice (Fig. 2B and Table 2). Since pathogenic auto-antibodies characteristic of lupus disease belong to the IgG2a and IgG3 isotypes, we measured these IgG subtypes in α -GalCer- and vehicle-treated, pristane-injected BALB/c mice. No significant differences were observed in any of these Ig subtypes (data not shown).

We have previously shown that marginal zone B cells, which express high levels of CD1d molecules and are enriched in autoreactive cells, are increased in pristane-injected BALB/c

mice [18]. We therefore evaluated the effects of α -GalCer treatment on the prevalence of marginal zone B cells. Results showed that α -GalCer treatment suppresses the prevalence of CD21^{high}CD23^{low}B220⁺ marginal zone B cells in the spleen of pristane-injected BALB/c mice (Fig. 2C).

We also examined the requirement of CD1d expression for the capacity of α -GalCer to protect BALB/c mice against pristane-induced lupus. Results showed that α -GalCer suppresses the development of proteinuria and anti-dsDNA antibodies in BALB/c.*CD1d*^{+/+} mice but not BALB/c.*CD1d*^{-/-} mice (Tables 1, 2).

Effects of repeated α -GalCer administration to pristane-injected BALB/c mice on NKT and conventional T cell responses

To evaluate the effects of repeated α -GalCer administration on immune responses of pristane-injected BALB/c mice, we examined NKT and conventional T cell responses of α -GalCer- and vehicle-treated mice at 9 and 12 months after pristane injection (Fig. 3 and data not shown). Consistent with prior studies in other mouse strains [21,22], repeated injection of BALB/c mice with α -GalCer resulted in a reduction in the prevalence of NKT cells and an increase in the expression of inhibitory Ly49 receptors by the remaining NKT cells, as compared with vehicle-injected mice (Fig. 3A). To test whether this diminution in the prevalence of NKT cells also results in defective NKT function, we stimulated spleen cells *in vitro* with α -GalCer and measured IFN- γ and IL-4 levels. We found that α -GalCer-injected mice generated reduced levels of both IFN- γ and IL-4 as compared with vehicle-treated mice (Fig. 3B), indicating that NKT cell functions are suppressed in α -GalCer-treated, pristane-injected animals.

To evaluate responses mediated by conventional T cells, spleen cells from pristane-injected mice treated with α -GalCer or vehicle were stimulated *in vitro* with plate-bound anti-CD3 antibodies, followed by measurement of IFN- γ and IL-4 secretion in the supernatant. Results showed no significant difference between the α -GalCer- and vehicle-treated groups (Fig. 3C).

α -GalCer-mediated prevention of pristane-induced lupus in BALB/c mice requires IL-4 expression

Prior studies evaluating the capacity of α -GalCer to prevent type 1 diabetes and EAE have provided evidence for a critical role for IL-4 in disease protection [23]. Therefore, we tested the capacity of α -GalCer to modulate pristane-induced SLE in BALB/c.*IL-4*^{-/-} mice. Results demonstrated that α -GalCer is unable to suppress proteinuria and anti-dsDNA levels in BALB/c.*IL-4*^{-/-} mice (Fig. 4 and Table 1). Instead, α -GalCer enhanced proteinuria levels in these animals, indicating disease exacerbation.

α -GalCer exacerbates pristane-induced lupus in SJL mice

Next, we tested the capacity of α -GalCer to modulate disease in pristane-injected SJL mice. In sharp contrast with results in BALB/c mice, we found that the proteinuria grades in α -GalCer-treated SJL mice, as compared with vehicle-treated mice, were significantly increased (Fig. 5A and Table 1). Analysis of lupus-related antibodies further revealed a trend for α -GalCer-induced increases in anti-dsDNA (Fig. 5B and Table 2) and IgG3 levels (Fig. 5C), although differences observed did not reach statistical significance in any single experiment. Interestingly, α -GalCer treatment of pristane-injected SJL mice resulted in expansion of marginal zone B cells (Fig. 5D).

Serositis (*i.e.* inflammation and fluid accumulation in serosal cavities such as the peritoneum) is a major manifestation of SLE in about 30–60% of patients [24]. This manifestation is not normally seen in pristane-injected BALB/c mice. We also did not observe the presence of ascites in vehicle-treated, pristane-injected SJL (*n*=36) or α -GalCer- or vehicle-treated,

pristane-injected BALB/c mice ($n=48$). Strikingly, however, 25% of α -GalCer-treated, pristane-injected SJL mice ($n=36$, pooled from three separate experiments) developed overt ascites. Evaluation of ascites fluid from three of these animals showed the presence of high levels of protein (3–13 mg/ml), suggesting its inflammatory nature. To further characterize the nature of inflammation, we collected peritoneal fluid from mice with or without ascites. We found that α -GalCer-treated, pristane-injected mice had increased prevalence of B220⁺ B cells and CD8⁺ T cells (data not shown), and increased levels of IL-6 and IL-12 (data not shown), two cytokines that have been reported to play a pathogenic role in pristane-induced lupus [25,26].

We also investigated whether the timing of α -GalCer treatment with respect to pristane injection influences the impact of α -GalCer treatment on disease development. For this purpose, we injected SJL mice with α -GalCer for a period of 1 month prior to, rather than after, pristane injection ($n=9$ –10 mice per group). This manipulation had no impact on disease parameters (data not shown). Further, we evaluated the capacity of α -GalCer to modulate disease when treatment was initiated 1 month after pristane injection ($n=10$ mice per group). Again, no significant effects on disease parameters were observed (data not shown). These findings suggest that α -GalCer impacts disease at the time of, but not prior to, or significantly after, disease induction.

Effects of repeated α -GalCer administration to pristane-injected SJL mice on immune responses

To evaluate effects of repeated α -GalCer treatment on immune responses of SJL mice, we analyzed NKT, conventional T and B cell responses in α -GalCer- and vehicle-treated mice at 6 months after pristane injection. Consistent with our studies in BALB/c mice (see Fig. 3A), repeated administration of α -GalCer resulted in profound NKT cell depletion and up-regulation of inhibitory Ly49 receptors (Fig. 6A). Surprisingly, however, *in vitro* restimulation of splenocytes from α -GalCer-treated mice with α -GalCer resulted in increased IFN- γ and relatively unaltered IL-4 responses, as compared with vehicle-treated mice (Fig. 6B).

To evaluate responses of conventional T cells, spleen cells from mice treated 6 months earlier with pristane and with a series of α -GalCer or vehicle injections, were stimulated with plate-bound anti-CD3 antibodies, followed by measurement of IFN- γ and IL-4 secretion in the supernatant. Results showed a trend for increased IFN- γ secretion in α -GalCer-treated mice, although results did not reach statistical significance (Fig. 6C and data not shown). We found no significant effects on the production of IL-4, which was barely detectable in these cultures.

To evaluate B cell responses, we stimulated spleen cells with LPS and measured total IgG and IgG subtype levels. Total IgG levels were similar in α -GalCer- and vehicle-treated mice (Fig. 6D). Interestingly, IgG2a levels were slightly increased in α -GalCer-treated mice (Fig. 6D), but no significant effects on IgG1 (Fig. 6D) and IgG3 (data not shown) levels were found. To evaluate cytokines that impact B cell function, we evaluated IL-6, TNF- α , and IL-12 levels in the same cultures. Results showed a slight increase in IL-6 but no significant difference in IL-12 or TNF- α production in α -GalCer-treated mice (Fig. 6E).

Discussion

SLE in humans is a heterogeneous disease that can differ in severity, temporal profile, autoantibody types and specificities, antibody deposition in tissues, and cytokine profiles [1]. Several inherited and induced mouse models of SLE that similarly differ in their severity, disease profile, and pathology, are now available. Disease induced by pristane is characterized by mesangial and focal kidney lesions [16], which is reminiscent of 50% of lupus patients

[24]. Therefore, the pristane-induced lupus model represents a complementary approach to the hereditary models for studying the pathogenesis of lupus and for testing therapeutic modalities.

Because NKT cells have been implicated in SLE, both in mouse models and in humans, and because the NKT cell ligand α -GalCer effectively protects susceptible mice against several organ-specific autoimmune diseases, we investigated the capacity of α -GalCer to modulate antibody-mediated disease in the pristane-induced model for SLE. We found that α -GalCer protects BALB/c mice against pristane-induced lupus-like auto-immunity, but exacerbates disease induced in SJL mice. We also found that α -GalCer induces a burst of IL-4 and IFN- γ production by NKT cells from BALB/c mice but induces little IL-4 production by NKT cells from SJL mice, providing a potential explanation for the dichotomous effects of NKT cell activation on the induction of lupus-like autoimmunity in these distinct genetic backgrounds. These findings indicate that NKTcell activation suppresses or promotes the progression of pristane-induced lupus, depending on the particular mouse strain in which disease is induced.

We have previously shown that CD1d deficiency in BALB/c mice exacerbates pristane-induced lupus nephritis, which correlated with reduced IL-4 production by T cells and expansion of marginal zone B cells [18]. Consistent with these findings, we here show that repeated α -GalCer injection in pristane-injected BALB/c mice protects against lupus nephritis, and this is associated with contraction of marginal zone B cells, as compared with vehicle-treated mice. Our studies with BALB/c.*IL-4*^{-/-} mice further showed that α -GalCer exacerbates disease in these animals, which demonstrates a critical role for IL-4 in the capacity of α -GalCer to prevent pristane-induced lupus. These findings are consistent with the idea that the pathogenesis of pristane-induced lupus is mediated by an exacerbated Th1 response. This conclusion is supported by previous studies indicating that pristane-induced lupus nephritis is suppressed in IFN- γ -[27], IL-6- [26], and IL-12p35-deficient [25] mice, but normal or slightly enhanced in IL-4-deficient mice [27]. Hence, the α -GalCer-mediated prevention of pristane-induced lupus in BALB/c mice is most likely due to the capacity of α -GalCer to promote Th2 deviation in these animals. Conversely, disease exacerbation in SJL mice is most likely due to α -GalCer-induced Th1 deviation.

While our studies demonstrated significant effects of α -GalCer treatment on proteinuria levels, no statistically significant differences were obtained for anti-dsDNA antibodies. Nevertheless, trends for anti-dsDNA antibodies correlated with the differences observed for proteinuria levels. Consistent with the modest effects on anti-dsDNA antibodies observed here, several recent studies have shown that anti-dsDNA antibody levels do not always correlate with clinical lupus disease [28–30].

A number of prior studies have provided evidence for a mechanistic link between the susceptibility of different mouse strains to lupus and the functional status of NKT cells. For example, B6-*lpr/lpr* and MRL-*lpr/lpr* mice have defective NKT cell numbers and functions [7], suggesting a protective role of NKT cells in lupus-like disease. Consistent with this idea, genetic ablation of CD1d in these animals exacerbated inflammatory dermatitis [31]. The reduced numbers and altered functions of NKT cells from SJL mice that we [20] (Fig. 1) and others [19] have observed may be responsible, at least in part, for the exquisite sensitivity of these animals to pristane-induced lupus. A similar mechanistic link has been proposed for the high susceptibility of SJL mice to EAE [20].

Interestingly, low numbers and suppressed functions of NKT cells have been observed in a variety of autoimmune-prone mouse strains, including non-obese diabetic (NOD) mice that are susceptible to type 1 diabetes [23]. However, the remaining NKT cells in NOD mice differ from those in SJL mice in that they produce substantial amounts of IL-4 in response to α -GalCer stimulation [23]. Consequently, repeated injection of NOD mice with α -GalCer promoted Th2

deviation and protected these animals against type 1 diabetes [23]. In the case of pristane-induced lupus in SJL mice, however, we found that α -GalCer exacerbates disease. The most likely explanation for these results is that NKT cells from SJL mice produce little IL-4, but substantial or even augmented amounts of IFN- γ , in response to α -GalCer stimulation. Consistent with our present findings, we have previously shown that α -GalCer is unable to prevent relapsing-remitting EAE in SJL mice, but instead had a tendency for enhancing disease severity and mortality in these animals [20]. Taken together, these findings illustrate that the effects of α -GalCer administration on the progression of autoimmunity are dependent upon the functional characteristics of the NKT cells of the particular mouse strain that is utilized.

α -GalCer holds significant promise for development as a therapeutic for autoimmune diseases [14]. Our present findings highlight the possibility that strain-dependent differences may influence the outcome of α -GalCer treatment on the development of Th1-mediated autoimmunity. It has previously been shown that patients with SLE have suppressed NKT cell numbers and functions [8–11], which may be similar to the functional status of these cells in SJL mice. Our findings further showed that disease exacerbation in SJL mice is related to a Th1 bias in the NKT cells of these animals. Because humans represent a heterogeneous outbred population, it remains possible that repeated α -GalCer treatment will promote Th2 responses in some individuals, but Th1 responses in others, depending on the genetic make-up of individuals and their autoimmune status. At present, it is therefore likely that α -GalCer will prevent Th1-mediated autoimmunity in some human subjects, while exacerbating or accelerating disease in others. Despite these potential drawbacks of α -GalCer treatment, it may be possible to manipulate the NKT cell response to selectively induce Th2 cytokine production, even in individuals with Th1-biased NKT cells [32].

Materials and methods

Mice

Female C57BL/6 (B6), BALB/cJ (BALB/c), SJL/J (SJL), and BALB/c.*IL-4*^{-/-} (BALB/c-*Il4*^{tm2Nnt}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *CD1d*^{-/-} mice from the ninth backcross to BALB/c have been described [18].

Reagents

Pristane was purchased from Sigma-Aldrich (St. Louis, MO). A synthetic form of α -GalCer, KRN7000 [33], was kindly provided by Kirin Brewery Co., Ltd. (Gunma, Japan). α -GalCer-loaded CD1d1 tetramers (CD1d tetramers) were prepared as described previously [34]. Anti-TCR β -FITC or -allophycocyanin, anti-NK1.1-PE or -PerCP-Cy5.5, anti-B220-PerCP, anti-CD3-PerCP, anti-CD23-FITC, anti-CD21-PE, anti-Ly49A-FITC, anti-Ly49C/I-FITC, anti-Ly49D-FITC, anti-Ly49G2-FITC, anti-IL-4-allophycocyanin, and anti-IFN- γ -FITC were obtained from BD Biosciences PharMingen (San Diego, CA).

Flow cytometry

Single-cell suspensions of spleen were prepared according to standard procedures and liver mononuclear cells were prepared as described [35]. NKT cells were identified by incubating cells with anti-CD16/32 antibodies (BD Biosciences PharMingen) to block FcR γ II/III receptors followed by staining with CD1d tetramers and anti-TCR β and anti-B220 antibodies, as described [34,35]. Cells were stained and analyzed by flow cytometry as described [35].

Activation of NKT cells, conventional T cells, and B cells

In vivo responses to α -GalCer were measured by injecting mice i.p. once or multiple times with 6 μ g α -GalCer suspended in 0.025% polysorbate-20 in PBS (vehicle) or with vehicle only.

Mice were bled at different time points and serum cytokine, Ig isotype, and IgG subtype levels were measured by ELISA or cytometric bead array (CBA). Alternatively, cells were stained for identification of different cell populations. NKT cells were activated *in vitro* by incubating spleen cells (2.5×10^5 per well) with titrated doses (1–100 ng/ml) of α -GalCer for 48–50 h, followed by measurement of [3 H]thymidine (PerkinElmer Life Sciences, Boston, MA) incorporation or cytokine production. For measurement of conventional T cell responses *in vitro*, splenocytes (2.5×10^5 per well) were stimulated with plate-bound anti-CD3 mAb (1–10 μ g/ml), supernatants were collected after 48 h of culture, and cytokines were measured. For measurement of B cell responses, spleen cells (2.5×10^5 per well) were stimulated with LPS (20 μ g/ml; Sigma-Aldrich), cytokines were measured 3 days later, and total IgG and IgG subtypes were measured 5 days later. Immune responses against protein antigens were evaluated as described [12].

Detection of cytokines, Ig isotypes, and IgG subtypes

Cytokine levels were measured using the mouse CBA kit (BD Biosciences PharMingen) following the manufacturer's instructions. Serum IgE levels were measured by a standard sandwich ELISA and IgG subtypes were measured by ELISA using an immunosubtyping kit (Southern Biotechnology, Birmingham, AL). Antigen-specific IgG1 and IgG2a levels were measured as described [12].

Establishment of pristane-induced lupus and α -GalCer treatment

Mice were injected i.p. once with 0.5 ml of pristane to induce lupus, as described [16]. In most experiments, mice were then injected with 6 μ g of α -GalCer or vehicle in 100 μ l of PBS, twice per week for 1 month, starting from the day of pristane administration. Mice were bled before and every 3 months after pristane administration, and sera were frozen for analysis of autoantibodies. All mice were monitored monthly for proteinuria and were sacrificed at 6, 9 or 12 months after pristane injection.

Assessment of lupus nephritis

Kidney disease was assessed in α -GalCer- and vehicle-treated BALB/c and SJL mice as described [36]. Proteinuria was measured as described [37] on a 0–4+ scale using a colorimetric assay strip for albumin (Albustix; Bayer Co., Elkhart, IN), where 0=absent, 1+ $\leq$ 30 mg/100 ml (mild), 2+=100 mg/100 ml (moderate), 3+=300 mg/100 ml, and 4+ $\geq$ 2,000 mg/100 ml (severe).

Detection of anti-double-stranded DNA antibodies

Anti-dsDNA antibody titers were measured by ELISA every 3 months after pristane injection, as described [36,37]. Anti-dsDNA antibody titers are expressed as U/ml using a reference standard of pooled serum from MRL-*lpr/lpr* mice.

Statistical analysis

Data are shown as mean values $\pm$ SE. Statistical analyses were performed using Student's *t*-test, Mann-Whitney *U*-test, or the two-sided Fisher's exact test.

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Abbreviations

α -GalCer

α -Galactosylceramide

dsDNA

Double-stranded DNA

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Fig. 1.

NKT cell numbers and functions in B6, BALB/c, and SJL mice. (A) CD1d tetramer⁺ cells in different mouse strains. (B) Serum cytokines after α -GalCer injection. Mice were injected i.p. with α -GalCer (6 μ g/mouse) or vehicle and bled at the indicated times for cytokine measurement. (C) Serum IgE levels after α -GalCer injection. Mice were injected with α -GalCer or vehicle on days 0, 4 and 7 and bled on day 11 for measurement of serum IgE. (D) *In vitro* cytokine responses of splenocytes to α -GalCer stimulation. Splenocytes from the indicated mice were stimulated with α -GalCer (100 ng/ml) and supernatants were collected after 48–50 h for measurement of IFN- γ and IL-4. (E) NKT cell cytokine production after α -GalCer injection. Spleen cells from the indicated mice were stimulated *ex vivo* with α -GalCer (100 ng/ml) or PMA (20 ng/ml) plus ionomycin (1 μ M) for 6 h, stained, and analyzed by flow cytometry. Data are shown for NKT cells (B220⁺ tetramer⁺ cells). (F, G) Modulation of adaptive immune responses by α -GalCer. Mice were immunized with HEL suspended in CFA, treated with vehicle or α -GalCer on days 0, 4, and 7, and HEL-specific T cell and antibody responses were

measured on day 11. Data shown are representative of three individual experiments with three to four mice per group. * $p < 0.05$.

Fig. 2.

α -GalCer protects BALB/c mice against pristane-induced SLE. BALB/c mice were injected with pristane and treated with α -GalCer (6 μ g/mouse, twice per week for 1 month) or vehicle. (A) Proteinuria grades at different time points after pristane injection ($n=23$ vehicle-injected and 25 α -GalCer-treated mice, pooled from four independent experiments). (B) Serum anti-dsDNA antibody levels from one representative of four experiments. (C) Marginal zone B cells at 12 months after pristane injection. * $p<0.05$.

Fig. 3. Effects of α -GalCer treatment on NKT and conventional T cell responses in pristane-injected BALB/c mice. BALB/c mice were injected with pristane and treated with α -GalCer or vehicle as described in the legend to Fig. 2. Mice were killed 12 months after pristane administration. (A) Prevalence of NKT cells and Ly49 expression. Data for Ly49s expression were gated on tetramer⁺ cells. (B, C) *In vitro* responses of splenocytes to α -GalCer (B) or anti-CD3 (C) stimulation. Splenocytes were stimulated with the indicated doses of α -GalCer or plate-bound anti-CD3, supernatants were collected after 48–50 h, and cytokines were measured. Results are representative of two independent experiments. * p <0.05.

Fig. 4. Requirement for IL-4 in the capacity of α -GalCer to protect BALB/c mice against pristane-induced lupus. BALB/c.*IL4*^{+/+} (WT) and BALB/c.*IL4*^{-/-} (IL-4 KO) mice were injected with pristane and treated with α -GalCer or vehicle as described in the legend to Fig. 2. (A) Proteinuria grades at different time points after pristane injection ($n=17$ vehicle-injected and 16 α -GalCer-treated mice, pooled from two independent experiments). (B) Serum anti-dsDNA antibody levels from a representative experiment. * $p<0.05$.

Fig. 5.

α -GalCer exacerbates pristane-induced SLE in SJL mice. SJL mice were injected with pristane and treated with α -GalCer or vehicle as described in the legend to Fig. 2. (A) Proteinuria grades at different time points after pristane injection ($n=36$ each of vehicle- and α -GalCer-treated mice, pooled from three independent experiments). (B) Serum anti-dsDNA antibody levels from a representative experiment. (C) Serum IgG3 antibody levels. (D) Marginal zone B cells at 6 months after pristane injection. * $p<0.05$.

Fig. 6.

Effects of α -GalCer treatment on NKT, conventional T, and B cell responses in pristane-injected SJL mice. SJL mice were injected with pristane and treated with α -GalCer or vehicle as described in the legend to Fig. 2. Mice were killed at 6 months after pristane administration. (A) Prevalence of NKT cells and Ly49 expression. (B, C) *In vitro* responses of splenocytes to α -GalCer (B) or anti-CD3 (C) stimulation. Splenocytes were stimulated with the indicated doses of α -GalCer or plate-bound anti-CD3, supernatants were collected after 48–50 h, and cytokines were measured. (D, E) Splenocyte responses to LPS. Splenocytes were incubated with medium or LPS (20 μ g/ml) and culture supernatants were collected after 5 or 3 days for

measurement of Ig (D) or cytokines (E), respectively. Results are representative of two independent experiments. * $p < 0.05$.

Table 1Effect of α -GalCer treatment on the cumulative frequency of proteinuria in BALB/c and SJL mice^{a)}

Mice	Months after pristane injection			
	3	6	9	12
BALB/c				
Vehicle (n=23)	30.4	71.4	87.5	100 ^{b)}
α -GalCer (n=25)	20.0	33.3 ^{**}	42.9 [*]	30.0 ^{b),*}
Balb/c. <i>CD1d</i> ^{-/-}				
Vehicle (n=12)	25.0	50.0	100	ND
α -GalCer (n=13)	30.8	69.2	100	ND
Balb/c. <i>IL-4</i> ^{-/-}				
Vehicle (n=17)	41.2	58.8	70.5	ND
α -GalCer (n=16)	18.7	75.0 [*]	100 [*]	ND
SJL				
Vehicle (n=36)	11.1	43.5	66.7 ^{b)}	ND
α -GalCer (n=36)	19.5	85.2 [*]	100 ^{b),*}	ND

^{a)} Mice were injected with pristane and treated with α -GalCer (6 μ g/injection/mouse) or vehicle, twice per week, starting from the day of pristane administration. Proteinuria grades were estimated at the indicated time points. Results are shown as the cumulative percentage of mice having moderate to severe (≥ 100 mg/100 ml or 2+) proteinuria.

^{*} $p < 0.05$;

^{**} $p < 0.005$; ND, not done.

^{b)} Results represent only one experiment, as other experiments were terminated prior to that time point.

Table 2

Effect of α -GalCer treatment on serum anti-dsDNA antibodies^{a)}

Treatment	n	Months after pristane injection				
		3	6	9	12	
BALB/c						
Exp 1	10	289381	1254±268	858±218	1473±518	
α -GalCer	10	245±102	1208±157	426±127	750±366	
Vehicle	8	ND	ND	271±110	538±123	
α -GalCer	10	ND	ND	113±55	427±58	
Vehicle	5	342±89	540±78	ND	ND	
α -GalCer	5	310±126	387±84	ND	ND	
BALB/c.CD1d ^{-/-}						
Exp 1	5	ND	524±91	4730±933	ND	
α -GalCer	5	ND	723±142	3225±917	ND	
Vehicle	7	105±26	247±50	ND	ND	
α -GalCer	8	110±23	361±103	ND	ND	
BALB/c.IL-4 ^{-/-}						
Exp 1	9	443±69	879±159	ND	ND	
α -GalCer	8	408±86	730±137	ND	ND	
Vehicle	8	485±156	539±112	834±150	ND	
α -GalCer	8	492±92	644±127	1287±247	ND	
SJL						
Exp 1	12	1705±374	1488±611	717±166	ND	
α -GalCer	12	1180±207	3393±1541	3887±2169	ND	
Vehicle	15	2678±523	1297±560	ND	ND	
α -GalCer	15	4075±751	1985±686	ND	ND	

a) Mice were injected with pristane and treated with α -GalCer (6 μ g/injection/mouse) or vehicle, twice per week, starting from the day of pristane administration. Anti-dsDNA levels were measured at the indicated time points and are expressed as U/ml. ND, not done.