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β-galactosylceramide alters invariant natural killer T cell function and is effective treatment for lupus☆

Sufi R. Morshed [a], Tsuyoshi Takahashi [a], Paul B. Savage [b], Neeraja Kambham [c], Samuel Strober [a,]*

[a] *Department of Medicine, Division of Immunology and Rheumatology, Stanford University School of Medicine, Stanford, CA 94305, USA*
[b] *Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602, USA*
[c] *Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA*

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Abstract NZB/W female mice spontaneously develop systemic lupus, an autoantibody mediated disease associated with immune complex glomerulonephritis. Natural killer (NK) T cells augment anti-dsDNA antibody secretion by NZB/W B cells in vitro, and blocking NKT cell activation in vivo with anti-CD1 mAb ameliorates lupus disease activity. In the current study, we show that β-galactosylceramide reduces the in vivo induction of serum IFN-γ and/or IL-4 by the potent NKT cell agonist α-galactosylceramide and reduces NKT cell helper activity for IgG secretion. Treatment of NZB/W mice with the β-galactosylceramide ameliorated lupus disease activity as judged by improvement in proteinuria, renal histopathology, IgG anti-dsDNA antibody formation, and survival. In conclusion, β-galactosylceramide, a glycolipid that reduces the cytokine secretion induced by a potent NKT cell agonist ameliorates lupus in NZB/W mice.
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Introduction

NZB/W female mice spontaneously develop systemic lupus erythematosus with immune complex glomerulonephritis appearing at about 6 months of age [1,2]. The onset of kidney disease is associated with the IgM to IgG isotype switching of autoantibodies to a variety of nucleic acids and associated proteins [1,2]. T cells play an important role in augmenting autoantibody secretion of B cells and in facilitating switching to the IgG2a pathogenic isotype [3,4,5]. The marked amelioration of disease activity after treatment of NZB/W mice with anti-CD4 monoclonal antibody (mAb) suggested an important contribution of $CD4^+$ T cells to the development of lupus [3]. In addition, $CD4^+$ T cells have been shown to augment spontaneous secretion of IgM, IgG, and autoantibodies by B cells in vitro [4,5]. CD1d reactive natural killer (NK) T cells have been shown to contribute to the development of lupus in NZB/W mice by helping B cells to secrete autoantibodies including IgG anti-

Abbreviations: NK, natural killer; dsDNA, double stranded DNA; α-GalCer, α-galactosylceramide; β-GalCer, β-galactosylceramide.

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* Corresponding author. Div of Immunology and Rheum/Medicine, Stanford University School of Medicine, CCSR Building, Room 2215-C, 269 Campus Dr., Stanford, CA 94305-5166, USA. Fax: +1 650 725 6104.
E-mail address: sstrober@stanford.edu (S. Strober).

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dsDNA antibodies in vitro [6,7]. Interestingly, conventional $CD4^+$ T cells failed to show helper activity in vitro under the culture conditions that were used [7]. The large majority of these NKT cells express CD4 and can recognize endogenous ligands associated with the CD1d antigen presenting molecule on B cells [7]. BALB/c mouse T cells with an anti-CD1d TCR transgene induced lupus in nu/nu BALB/c mice after adoptive transfer [8].

Although some CD1d binding glycolipids such as α-galactosylceramide are potent activators of NKT cells and induce robust secretion of IL-4 and IFN-γ in the serum, a single parenteral injection of β-galactosylceramide (β-GalCer) with a 12 carbon acyl chain (C12) has been reported to cause a rapid reduction of the percentage of NKT cells in the liver and spleen without the appearance of IL-4 and IFN-γ in the serum of normal C57BL/6 mice [9]. In the current study, we determined whether β-GalCer (C12) was able to reduce the NKT cell cytokine secretion induced by the potent NKT cell activator α-GalCer (C26), and whether β-GalCer (C12) treatment of NZB/W mice can ameliorate lupus.

Materials and methods

Mice

NZB/W and C57BL/6 female mice were purchased from the Jackson Laboratories (Bar Harbor, ME), and were maintained in the Stanford University Department of Comparative Animal Medicine until they were used for experiments at 10–12 weeks of age, unless otherwise stated in the text. All animal protocols were reviewed and approved by the Stanford Administrative Panels on Laboratory Animal Care.

Reagents and monoclonal antibodies (mAbs)

Anti-mouse mAbs including FITC-anti-CD5 (53–7.3), FITC-anti-CD21 (7G6), PE-anti-CD23 (B3B4), APC-anti-B220 (RA3–6B2), APC-anti-TCRβ (H57–597), and mouse CD1d-dimers were purchased from BD Biosciences (San Diego, CA). PE-conjugated PBS-57 glycolipid-loaded CD1d tetramers were obtained from the NIH Tetramer Facility, Rockville MD. αGalCer (C26) and αGalCer (C8) were kindly provided by Dr. Paul B. Savage (Brigham Young University, UT). Biochemical synthesis of these compounds has been described in detail previously [10]. β-GalCer (C12) was purchased from the Avanti Polar Lipid Company (Alabaster, AL). The procedure for loading of CD1d-dimers with glycolipids has been reported [6].

Administration of glycolipids

Lyophilized glycolipids were diluted in distilled water to make stock solutions of 200 μg/ml, and then further diluted in phosphate buffered saline to achieve the desired dose in 0.5 ml for intraperitoneal injections. Administration of β-GalCer by gavage was performed thrice a week at the dose of 800 μg/ml in 0.5 ml of phosphate buffered saline.

Cell preparation and immunofluorescent staining

Single-cell suspensions were prepared from livers, filtered through a fine nitex membrane, overlaid on lympholyte-M (Cedarlane, Ontario, Canada), separated by density centrifugation, and then washed with MACS buffer. Stainings were performed in the presence of anti-CD16/32 mAb (2.4G2, Pharmingen) at saturation to block FcRII/III receptors. Propidium iodide (Sigma Chemicals, St Louis, MO) was added to staining reagents to exclude dead cells. Staining for CD1 on liver and spleen cells used biotinylated anti-CD1 antibodies and PE-streptavidin for counterstaining. Single-cell suspensions from spleens were washed twice, and filtered through a fine nitex membrane. Cells were incubated with various combinations of mAbs, and two- to three-color immunostaining and flow cytometry were performed using standard techniques and equipment (LSR, BD Biosciences, San Jose, CA) with FlowJo software (TreeStar, Ashland, OR) for data analysis [6,7]. Four mice were used per experiment.

ELISA assays

A standard sandwich ELISA was used to measure concentrations of IgM, total IgG, IgG1, and IgG2a in serum and cell culture supernatant as described previously [6–8]. Reagents for the ELISA were purchased from BD Bioscience. Measurement of IL-4 or IFNγ was in an ELISA kit according to the manufacturer's protocol (BD Biosciences). IgM or IgG anti-dsDNA antibodies were captured using deproteinized calf thymus DNA, and isotype specific anti-globulin reagents were used in an ELISA as described in detail [6–8]. Anti-dsDNA antibody titers are expressed in units per milliliter, using a reference-positive standard of pooled serum from 9-month-old NZB/W mice. A 1:100 dilution of this standard serum was arbitrarily assigned a value of 100 U/ml as described before [6–8].

In vitro cultures of NKT cells and B-1 B cells

NKT cells were sorted by flow cytometry using CD1d-tetramers for staining, and B-1 B cells were sorted on the basis of the $CD19^+CD5^+CD23^-$ phenotype as described in detail previously [7]. The purified B-1 B cells were either cultured alone (1×10^5 cells/well) or together with purified NKT cells (5×10^4 cells/well) in 96-well round bottom plates for 10 days in complete medium [7]. Supernatants were harvested at the end of the culture period from triplicate wells, and the concentrations of IgM, IgG1, and IgG2a were determined by ELISA [7].

Monitoring lupus disease activity

Proteinuria of female NZB/W mice was measured weekly on a scale of 1–4^+ using a colorimetric assay for albumin (Albustix; Miles Inc., Elkhart, IN). Mice were considered to have proteinuria if at least two consecutive urine samples were greater than 2+, according to the scale (100 mg/dl). Blood samples were harvested monthly starting at 10 weeks of age and serum concentrations of total IgG and IgG-anti-dsDNA antibodies were determined by ELISA as described above. Survival of mice was monitored daily, and moribund mice were

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