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Aberrant Genetic Control of Invariant TCR-Bearing NKT Cell Function in New Zealand Mouse Strains: Possible Involvement in Systemic Lupus Erythematosus Pathogenesis

Kazuyuki Tsukamoto,* Mareki Ohtsuji,* Wakana Shiroiwa,∗† Qingshun Lin,∗† Kazuhiro Nakamura,* Hiromichi Tsurui,* Yi Jiang,§ Katsuko Sudo,‡ Hiroyuki Nishimura,§ Toshikazu Shirai,* and Sachiko Hirose‡∗

Both suppressive and promoting roles of NKT cells have been reported in the pathogenesis of systemic lupus erythematosus (SLE). Herein, we found that although New Zealand mice have normal frequencies of NKT cells, their in vitro potential to produce IL-4 and IFN-γ in response to α-galactosylceramide was remarkably impaired in New Zealand Black (NZB) mice prone to mild SLE, while production was highly up-regulated in nonautoimmune New Zealand White (NZW) mice and at intermediate levels in (NZB × NZW)F1 mice, which are prone to severe SLE. Because this aberration is evident in young mice before disease onset, genetic mechanisms are thought to be involved. Genome-wide quantitative trait locus analysis and association studies revealed that a locus linked to D11Mit14 on chromosome 11 may be involved in the difference in cytokine-producing potential between NZB and NZW NKT cells. Additionally, (NZB × NZW)F1 × NZB backcross progeny with the NZW genotype for D11Mit14 showed significantly increased frequencies of age-associated SLE phenotypes, such as high serum levels of IgG, IgG anti-DNA Abs, and lupus nephritis. In coculture studies, α-galactosylceramide-stimulated NKT cells from NZW and (NZB × NZW)F1 mice, but not from NZB mice, showed significantly enhanced Ig synthesis by B cells. These findings suggest that the D11Mit14-linked NZW locus may contribute to the development of SLE in (NZB × NZW)F1 mice through a mechanism that up-regulates NKT cell function. Thus, this NZW allele may be a candidate of the NZW modifiers that act to promote (NZB × NZW)F1 disease. The Journal of Immunology, 2008, 180: 4530–4539.

N atural killer T cells belong to a lymphocyte lineage distinct from conventional T cells and bear both the NK cell marker NK1.1 and the conventional T cell marker CD3. Most human and mouse NKT cells express invariant TCR α-chain (Vα24Jα15 in humans and Vα14Jα281 in mice) (1–5) and are strongly activated in vivo and in vitro by glycolipid Ags such as α-galactosylceramide (α-GalCer) (3) presented by CD1d, a nonclassical MHC class I-like Ag-presenting molecule expressed on the surface of APCs (6, 7). When activated, NKT cells rapidly produce large amounts of various cytokines and trigger the activation of a variety of immune cells (1–5).

Accumulated evidence has shown that the number and function of NKT cells are reduced in patients with various autoimmune diseases, including type I diabetes (8, 9), systemic sclerosis (10–12), rheumatoid arthritis (11, 12), multiple sclerosis (13, 14), and systemic lupus erythematosus (SLE) (11, 12), suggesting that NKT cells play a suppressive role in autoimmune diseases. There are also publications that favor this hypothesis, reporting that type I diabetes-prone NOD mice have an intrinsic defect in the number and function of NKT cells, and that this defect is associated with disease development (15–17). Thus, the activation of NKT cells by repeated injections of α-GalCer or the introduction of transgenic Vα14 prevents spontaneous diabetes in NOD mice (18–20). Conversely, the absence of NKT cells in CD1d-deficient NOD mice increases the incidence of diabetes (21, 22). In MRL/lpr mice, invariant NKT cells are specifically reduced with aging (23), and repeated injections of α-GalCer prevent inflammatory dermatitis in these mice (24).

Activation of NKT cells by α-GalCer, or its derivative OCH, also protects against experimental autoimmune encephalomyelitis (25, 26) and collagen-induced arthritis (CIA) (27). However, the disease protection conferred by these glycolipids is not simply due to the increase in the number of NKT cells, but it correlates with their ability to suppress Th1 cell responses and/or to promote Th2 cell responses (25–27). Importantly, there are reports indicating that the incidence and the severity of CIA were reduced in NKT cell-deficient mice, as compared with findings in wild-type mice (27, 28). These observations are in contrast to those found in NKT cell-deficient NOD mice (23, 24). Thus, it was suggested that NKT cells function as not only suppressor but also effector cells in different types of autoimmune diseases.

*Department of Pathology and †Department of Obstetrics and Gynecology, Juntendo University School of Medicine; 2Animal Research Center, Tokyo Medical University, Tokyo; 3Toin Human Science and Technology Center, Department of Biomedical Engineering, Toin University of Yokohama, Yokohama, Japan; and 4Central Laboratory of First Clinical College, China Medical University, Shenyang, China

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2 Address correspondence and reprint requests to Dr. Sachiko Hirose, Department of Pathology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail address: sacchi@med.juntendo.ac.jp

3 Abbreviations used in this paper: α-GalCer, α-galactosylceramide; SLE, systemic lupus erythematosus; B6, C57BL/6; NZB, New Zealand Black; NZW, New Zealand White; CIA, collagen-induced arthritis; QTL, quantitative trait loci; LOD, logarithm of odds; Ntm1, NKT cell modifier 1; Tim, T cell Ig mucine.

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In this context, the evidence of the role of NKT cells in SLE-prone (New Zealand Black (NZB) × New Zealand White (NZW))F1 mice has been conflicting, with both suppressive and promoting roles having been reported (23, 29–32). As in cases of patients with SLE (11, 12), the age-associated reduction of NKT cells was reported to occur in (NZB × NZW)F1 mice (23). However, there are also reports showing that the age-associated progression of (NZB × NZW)F1 disease is associated with an expansion and activation of NKT cells (30, 32). Furthermore, the activation of NKT cells by repeated injections of α-GalCer in (NZB × NZW)F1 mice was shown to exacerbate lupus nephritis (31), while treatment of (NZB × NZW)F1 mice with anti-CD1d-blocking mAb ameliorated the disease (29, 31), suggesting that the role of NKT cells in these mice is disease promoting.

Early onset of severe SLE occurs in (NZB × NZW)F1 mice under the control of multiple susceptibility and modifying alleles derived from both parental NZB and NZW strains (33). NZB mice are prone to autoimmune hemolytic anemia, and they also develop SLE, but the disease phenotypes are mild and develop much later in life compared with (NZB × NZW)F1 mice. The NZW strain is principally a nonautoimmune strain, but it carries modifying genes to exacerbate SLE in the (NZB × NZW)F1 hybrid. Our present study shows evidence that the potential of invariant TCR-bearing NKT cells to produce IL-4 and IFN-γ in response to α-GalCer is remarkably impaired in NZB mice, while such potential is highly up-regulated in NZW mice. This aberrant potential of NKT cells is genetically controlled, and the genetic element of NZW with the high producing phenotype is suggested to contribute to the exacerbation of SLE in the F1 hybrid of NZB and NZW mice.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, NZB, NZW, and (NZB × NZW)F1 mice, originally obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained in our animal facility. All mice were housed under identical conditions, and all experiments were performed in accordance with institutional guidelines. Female mice were used in this study.

Cell preparation

Single-cell suspensions from the spleen and liver were prepared by gently grinding the organs between the frosted ends of glass slides and filtering them through a 100-μm nylon mesh in RPMI 1640 (Sigma-Aldrich) containing 2% FCS (JRH Biosciences). Liver cells were subsequently resuspended in 40% isotonic Percoll solution, gently overlayed on 80% isotonic Percoll solution, and centrifuged for 25 min at 2800 rpm at room temperature. Mononuclear cells were collected from the interface and washed once. RBC were lysed with an ammonium chloride lysis buffer and then washed once.

Flow cytometric analysis

To identify invariant TCR-bearing NKT cells, we used α-GalCer-loaded CD1d:Ig dimeric protein (Dimer XI, BD Pharmingen) consisting of the extra-cellular MHC class I-like domains of the mouse CD1d molecule fused with the V_{α} regions of mouse IgG1. To load α-GalCer onto the CD1d:Ig, the α-GalCer solution in DMSO was mixed with CD1d:Ig at 40 μM excess of α-GalCer, and incubated at 37°C for 24 h. Aliquots of cells suspended in normal goat serum were incubated with α-GalCer-loaded CD1d:Ig at a final concentration of 0.1 μg/ml CD1d:Ig in 6.8% DMSO on ice for 30 min. Cells were washed once and stained with FITC-conjugated goat anti-mouse IgG on ice for 30 min. After washing and blocking nonspecific IgG-binding with 2-4G2 mAb, cells were further stained with allopurinol-cyanin-conjugated anti-CD3 (2C11) mAb on ice for 30 min. For three-color flow cytometric analysis, cells were additionally stained with PE-conjugated anti-CD11c (HL3), anti-CD20 (RA3.682), PE-conjugated anti-NK1.1, or Alexa Fluor 488-conjugated anti-CD3 (1B1) mAbs on ice for 30 min. After the cells were washed and fixed in PBS containing 0.2% BSA and 1% formaldehyde, stained cells in the lymphocyte gate, as determined by forward and side scattering, were analyzed using a FACStar flow cytometer and CellQuest software (BD Biosciences). α-GalCer was obtained from the Kirin Pharmaceutical Research Institute. Abs for flow cytometric analysis were all purchased from BD Pharmingen.

Isolation of lymphocyte subsets

Liver NKT cells were obtained by sorting α-GalCer-CD1d-CD3+ NKT cells using a FACSARia cell sorter (BD Biosciences). Splenic T cells were obtained by positive sorting of α-GalCer-CD1d-CD3+ cells. T cell-depleted spleen cells (NKT and B cells) and NKT cell-depleted spleen cells (T and B cells) were obtained by negative sorting of α-GalCer-CD1d-CD3+ T cells and α-GalCer-CD1d-CD3+ NKT cells, respectively.

Measurement of Ag-presenting potential

The α-GalCer-presenting potential of splenic APCs was examined using NKT cell hybridoma, N38-3C3 (34). Spleen cells were treated with a mixture of rabbit complement (Cedarlane Laboratories) and Abs to Thyl.2 (J11), CD4 (GR1.5), CD8 (53-6.7), and asialoGM1 (Wako Pure Chemical) to remove T, NK, and NKT cells and serve as a source of APCs. A mixture of 5 × 10^6 cells/well of APCs and 2 × 10^5 cells/well of hybridoma cells was cultured in a 96-well round-bottom plate for 2 days in RPMI 1640 culture medium supplemented with 10% FCS, 45 μM 2-ME (Sigma-Aldrich), 100 U/ml penicillin (Meiji Seika Kaisha), and 100 μg/ml streptomycin (Meiji Seika Kaisha) in the presence of several concentrations of α-GalCer. The potential for α-GalCer presentation was estimated by the measurement of IL-2 secreted into the culture supernatant by ELISA, as described below.

Assays for cytokine and Ig production

An aliquot of ~1 × 10^6 cells/well, unless otherwise stated, was cultured in vitro in 96-well flat-bottom plates in RPMI 1640 culture medium for 2 days (for IL-4 and IFN-γ production) or 4 days (for Ig production). Cells were stimulated in a plate precoated with 10 μg/ml anti-CD3 (145–2C11) mAb or anti-CD3 and anti-CD28 (37.51) mAbs, or with a given concentration of α-GalCer. Levels of IL-2, IL-4, and IFN-γ in the culture supernatants were measured using standard sandwich ELISA with coated capture mAbs and biotinylated detection mAbs, according to the manufacturer’s instructions (BD Pharmingen). Ig levels and anti-DNA Ab levels were measured using ELISA. Mouse IgG and IgM standards were purchased from Rockland. DNA-binding activities were expressed in units, referring to a standard curve obtained by serial dilution of a standard serum pool from (NZB × NZW)F1 for 8 mo of age, containing 1000 U activities/ml.

Measurement of proteinuria

The severity of renal disease was monitored by biweekly testing for proteinuria, as described previously (35). Proteinuria was scored from grade 0 to 6, according to the amount of urinary albumin, in which 0 indicates <37 mg/100 ml; 1, ≥37 mg/100 ml; 2, ≥74 mg/ml; 3, ≥111 mg/100 ml; 4, ≥333 mg/100 ml; 5, ≥1000 mg/100 ml; and 6, ≥3000 mg/100 ml. Mice with urinary protein levels of 111 mg/100 ml or more in repeated tests were considered as being positive for proteinuria (35).

H-2 typing

H-2 typing was done by flow cytometry analysis using peripheral lymphocytes stained with FITC-conjugated anti-I-A* (Ia7) and anti-I-A^d (m52) mAbs.

Genotyping

DNA was extracted from mouse-tail tissue. Genotyping for microsatellite markers was done using PCR. Microsatellite primers were purchased from Research Genetics. PCR reactions were run in a total volume of 20 μl containing 40 ng of genomic DNA. A three-temperature PCR protocol (94°C, 65°C, and 72°C) was used for 35 cycles in a GeneAmp 9600 thermal cycler (PerkinElmer-Cetus). PCR products were diluted 2-fold with loading buffer consisting of xylene cyanol and bromophenol blue dyes in 50% glycerin and were run on 18% polyacrylamide gels. After electrophoresis, bands were visualized by ethidium bromide staining.

Statistics

For analyses of mouse strain differences in the amounts of cytokines produced by NKT cells and of immunoglobulins by B cells, cell samples obtained from five to six mice from each strain were tested in three or five experiments, and the differences in the mean amounts ± SEM between mouse strains were analyzed by using the Mann-Whitney U test. In studies of backcross mice, the statistical analysis was also done using the Mann-Whitney U test (p < 0.05) that was considered to be statistically significant. To estimate the position of quantitative trait loci (QTL) for the amount of cytokine produced in vitro by α-GalCer-stimulated spleen cells, the likelihood ratio statistics were determined using the Map Manager QT package program. Logarithm of odds
(LOD) scores of ≥3.3 were used as the threshold for statistically significant linkage. In addition to the criteria using the fixed level of LOD, a permutation test for a significance level of 1% was also performed in 1-centiMorgan (cM) steps for 1000 permutations using the Map Manager QT package program to further estimate the significance levels for QTL analysis.

**Results**

**Comparisons of the frequency and number of NKT cells among mouse strains**

Frequencies of NKT cells in the livers and spleens of 2-mo-old NZB, NZW, (NZB × NZW) (BW) F1, B6, and BALB/c mice. A, Double staining of liver mononuclear cells and spleen cells with anti-CD3 and α-GalCer-CD1d dimer or with anti-CD3 and anti-NK1.1 mAbs. Numbers show percentages of α-GalCer-CD1d⁺CD3⁺ NKT cells or NK1.1⁺CD3⁺ NKT cells per total of mononuclear cells shown in circles. Results are representative of five independent experiments. B, Analysis of α-GalCer-CD1d and NK1.1 expression on the surface of NK1.1⁺ NKT cells and α-GalCer-CD1d⁺ NKT cells, respectively. Liver mononuclear cells and spleen cells were triple stained with α-GalCer-CD1d, anti-CD3, and anti-NK1.1 mAbs, and α-GalCer-CD1d expression on NK1.1⁺CD3⁺ NKT cells and NK1.1 expression on α-GalCer-CD1d⁺CD3⁺ NKT cells were examined using histograms. The shaded histogram indicates the background staining. Numbers show percentage of α-GalCer-CD1d⁺ and NK1.1⁺ cells. Results are representative of three independent experiments.

**FIGURE 1.** Representative flow cytometric analysis of NKT cells from livers and spleens of 2-mo-old NZB, NZW, (NZB × NZW) (BW) F1, B6, and BALB/c mice. A, Double staining of liver mononuclear cells and spleen cells with anti-CD3 and α-GalCer-CD1d dimer or with anti-CD3 and anti-NK1.1 mAbs. Numbers show percentages of α-GalCer-CD1d⁺CD3⁺ NKT cells or NK1.1⁺CD3⁺ NKT cells per total of mononuclear cells shown in circles. Results are representative of five independent experiments. B, Analysis of α-GalCer-CD1d and NK1.1 expression on the surface of NK1.1⁺ NKT cells and α-GalCer-CD1d⁺ NKT cells, respectively. Liver mononuclear cells and spleen cells were triple stained with α-GalCer-CD1d, anti-CD3, and anti-NK1.1 mAbs, and α-GalCer-CD1d expression on NK1.1⁺CD3⁺ NKT cells and NK1.1 expression on α-GalCer-CD1d⁺CD3⁺ NKT cells were examined using histograms. The shaded histogram indicates the background staining. Numbers show percentage of α-GalCer-CD1d⁺ and NK1.1⁺ cells. Results are representative of three independent experiments.

Frequencies of NKT cells in the livers and spleens of 2-mo-old NZB, NZW, (NZB × NZW)F1, B6, and BALB/c mice were determined by double staining mononuclear cells with a mixture of anti-CD3 mAb and α-GalCer-loaded CD1d dimer or a mixture of anti-CD3 and anti-NK1.1 mAbs. The frequencies of α-GalCer-CD1d⁺CD3⁺ or NK1.1⁺CD3⁺ NKT cells were higher in the liver as compared with those seen in the spleen (BALB/c mice are negative for the NK1.1 allele) (Fig. 1A). The NKT cell frequencies in the livers of NZW and (NZB × NZW)F1 mice tended to be lower than those in other mouse strains; however, there was no statistically significant strain difference in α-GalCer-CD1d⁺CD3⁺ NKT cell frequencies in either spleens or livers (Table I). In flow cytometric analysis using cells triple stained with anti-CD3 mAb, anti-NK1.1 mAb, and α-GalCer-CD1d dimer, >80% of the NK1.1⁺CD3⁺ NKT cells in the livers and spleens of NZB, NZW, and (NZB × NZW)F1 mice were α-GalCer-CD1d⁺ (Fig. 1B), and >90% of α-GalCer-CD1d⁺CD3⁺ NKT cells in livers.
expressed high levels of NK1.1 Ag. A notable finding was that although >80% of α-GalCer-CD1d+CD3+ NKT cells in spleens were also NK1.1+, the expression levels of NK1.1 Ag were remarkably reduced in spleens, as compared with those in livers (Fig. 1B), and the findings were consistent with a previous report (32).

We then examined the age-associated changes in the frequency of α-GalCer-CD1d+CD3+ NKT cells in New Zealand mouse strains. As shown in Table I, comparisons of NKT cell frequencies between 2- and 8-mo-old NZB, NZW, and (NZB × NZW)F1 mice showed that there were no age-associated changes in either livers or spleens. However, when the total number of NKT cells in spleens was compared, a significant age-associated increase was observed in enlarged spleens from older NZB and (NZB × NZW)F1 mice.

Comparisons of cytokine-producing potential of NKT cells among mouse strains

We then compared the in vitro cytokine-producing potential of spleen cells upon stimulation with varying concentrations of α-GalCer among 2-mo-old NZB, NZW, (NZB × NZW)F1, B6, and BALB/c mice. As shown in Fig. 2A, spleen cells responded to

![Figure 2: Comparison of in vitro IL-4- and IFN-γ-producing potential of α-GalCer-stimulated spleen cells among mouse strains. A. Comparison of the potential among 2-mo-old mice. Whole spleen cells in triplicate cultures were cultured for 2 days in the presence of varying concentrations of α-GalCer, and the concentrations of IL-4 and IFN-γ in the culture supernatants were measured by ELISA. The mean amounts ± SEM of cytokines show data obtained from 5–6 mice from each strain in triplicate experiments. ***, p < 0.001; **, p < 0.01; and *, p < 0.05. B. Spleen cells from 2-mo-old mice were cultured for 2 days in the presence of 30 ng/ml α-GalCer, and the concentrations of IL-4 and IFN-γ in the culture supernatants were measured by ELISA. The mean amounts ± SEM of cytokines show data obtained from 5–6 mice from each strain in triplicate experiments. ***, p < 0.001; **, p < 0.01; and *, p < 0.05. C. Age-associated changes in cytokine producing potential of α-GalCer-stimulated spleen cells. Whole spleen cells from 2- and 6-mo-old NZB, NZW, and BW F1 mice were cultured for 2 days in the presence of 30 ng/ml α-GalCer, and the concentrations of IL-4 and IFN-γ in the culture supernatants were measured by ELISA. The mean amounts ± SEM of cytokines show data obtained from 5–6 mice from each strain at 2 and 6 mo of age in triplicate experiments. *, p < 0.05.

Table I. Frequency and number of α-GalCer-CD1d+CD3+ NKT cells

<table>
<thead>
<tr>
<th></th>
<th>NZB</th>
<th>NZW</th>
<th>BW F1</th>
<th>B6</th>
<th>BALB/c</th>
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<tbody>
<tr>
<td><strong>Frequency in livers (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-mo-old</td>
<td>27.8 ± 2.3</td>
<td>20.3 ± 6.2</td>
<td>23.3 ± 3.7</td>
<td>29.1 ± 1.0</td>
<td>29.0 ± 2.0</td>
</tr>
<tr>
<td>8-mo-old</td>
<td>31.1 ± 9.9</td>
<td>21.2 ± 0.3</td>
<td>24.5 ± 0.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Frequency in spleens (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-mo-old</td>
<td>1.4 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>8-mo-old</td>
<td>2.4 ± 0.4</td>
<td>2.4 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total NKT cell number in spleens (×10⁵)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-mo-old</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>1.6 ± 0.5</td>
<td>2.1 ± 0.3</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>8-mo-old</td>
<td>4.3 ± 0.6a</td>
<td>2.4 ± 0.8</td>
<td>5.5 ± 1.9a</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

Data show mean ± SEM of 5–8 mice. *Significantly increased compared to the number of 2-mo-old mice (p < 0.05).
α-GalCer in a dose-dependent manner; however, levels of the potential to produce IL-4 and IFN-γ varied considerably among the mouse strains. Spleen cells from BALB/c mice produced considerable amounts of both IL-4 and IFN-γ, while those from B6 mice produced a significant amount of IFN-γ but a minimal amount of IL-4. Intriguingly, compared with the findings in B6 and BALB/c mice, spleen cells from NZW mice produced larger amounts of both IL-4 and IFN-γ. In contrast, NZB spleen cells produced minimal amounts of both IL-4 and IFN-γ, and the amounts of IL-4 and IFN-γ produced by (NZB × NZW)F1 spleen cells were intermediate between the parental NZB and NZW mice. Fig. 2B compares IL-4- and IFN-γ-producing potentials of spleen cells stimulated with 30 ng/ml α-GalCer among mouse strains. NZW spleen cells produced significantly larger amounts of IL-4 and IFN-γ than those from other mouse strains tested. In contrast, NZB spleen cells produced significantly smaller amounts of both cytokines, as compared with those from NZW, (NZB × NZW)F1, and BALB/c mice.

We next examined age-associated changes in cytokine-producing potential of α-GalCer-stimulated spleen cells from NZB, NZW, and (NZB × NZW)F1 mice (Fig. 2C). Although spleen cells from NZB and NZW mice showed no significant changes, there was a significant age-associated increase in potential to produce both IL-4 and IFN-γ by spleen cells from (NZB × NZW)F1 mice.

To see whether such aberrant cytokine-producing potential of α-GalCer-stimulated spleen cells from NZB and NZW mice is indeed attributed to NKT, but not T cells, T cell-depleted spleen cells were stimulated in vitro in the presence of α-GalCer, and the amounts of IL-4 and IFN-γ produced were examined. As shown in Fig. 3A, while T cell-depleted spleen cells from NZW mice

FIGURE 3. Evidence that aberration in cytokine-producing potential of α-GalCer-stimulated spleen and liver cells from New Zealand mice is attributed to NKT cells per se. A, Comparison of in vitro IL-4- and IFN-γ-producing potential of α-GalCer-stimulated T cell-depleted spleen cells and anti-CD3 mAb-stimulated FACS-sorted splenic T cells between NZB and NZW mice. Spleen cells from 2-mo-old mice were stained with α-GalCer-CD1d and anti-CD3 mAb, and T cell-depleted spleen cells and splenic T cells were obtained by negative and positive sorting of α-GalCer-CD1d CT3 + T cells, respectively. The purity of the cells was 99% in these experiments. Sorted cells (1 × 10^6 cells/well) were cultured for 2 days in the presence of α-GalCer (30 ng/ml) or immobilized anti-CD3 mAb (10 μg/ml), and the concentrations of IL-4 and IFN-γ in the culture supernatants were measured by ELISA. The mean amounts ± SEM of cytokines show data from 5 mice from each strain in five experiments. **, p < 0.001; and *, p < 0.01. B, Comparison of cytokine-producing potential of flow cytometry-sorted liver NKT cells between NZB and NZW mice in response to immobilized anti-CD3 mAb and to anti-CD3 plus anti-CD28 mAbs. Liver NKT cells were obtained by positive sorting of α-GalCer-CD1d + CD3 + NKT cells from 2-mo-old NZB and NZW mice. The purity of the cells was >96% in both NZB and NZW experiments. Flow cytometry-sorted cells (2 × 10^5 cells/well) were cultured for 2 days in a plate precoated with anti-CD3 mAb (10 μg/ml) or with anti-CD3 and anti-CD28 (10 μg/ml) mAbs, and the concentrations of IL-4 and IFN-γ in the culture supernatants were measured by ELISA. The mean amounts ± SEM of cytokines show data from 5 mice from each strain in five experiments. ***, p < 0.001; and *, p < 0.01. C, Comparison of CD1d expression levels on the surface of splenic CD3 + T cells, B220 + B cells, CD11b + macrophages, and CD11c + dendritic cells by histogram between 2-mo-old NZB and NZW mice. Results are representative of three independent experiments. The shaded histogram indicates the background staining. D, Comparison of α-GalCer-presenting potential of APCs to NKT hybridoma cells (N38-3C3) between NZB and NZW mice. APCs were prepared from spleen cells of 2-mo-old mice by complement-dependent lysis of cells using Abs to Thy1.1, CD4, CD8, and asialoGM1, and cultured with or without NKT hybridoma cells (N38-3C3) in the presence of α-GalCer. α-GalCer-presenting potential was estimated by measurement of IL-2 produced by NKT hybridoma cells in culture. The concentration of IL-2 was measured by ELISA. The mean amounts ± SEM of cytokines show data from 5–6 mice from each strain in triplicate experiments.
produced significant amounts of both cytokines, only limited amounts of both cytokines were produced by those from NZB mice. Fig. 3A also shows the potential of FACS-sorted T cells from NZB and NZW mice to produce IL-4 and IFN-γ in response to immobilized anti-CD3 mAb. As for the production of IL-4, a significant amount was produced by NZW T cells and a limited amount by NZB T cells, similar to the profile of IL-4 synthesis by NKT cells. However, the IFN-γ-producing potential of T cells differed markedly from that of NKT cells, in which NZB T cells produced comparable amounts of IFN-γ as NZW T cells did.

Because the functional deficiency of NKT cells from NZB mice was remarkable, we then examined whether the functional defect in NZB NKT cells is due to NKT cells per se or to the impaired Ag-presenting potential of CD1d-bearing APCs. To exclude the effect of APCs, we first sorted CD1d-GalCer-CD1d CD3 liver NKT cells, stimulated with immobilized anti-CD3 mAb alone or together with mAb against costimulatory molecule CD28, and measured the amounts of IL-4 and IFN-γ in the culture supernatants. As shown in Fig. 3B, NZB NKT cells produced minimal amounts of both IL-4 and IFN-γ even in the presence of anti-CD3 and anti-CD28 mAbs, compared with NZW NKT cells that produced large amounts of both cytokines, suggesting that the functional deficiency of NZB NKT cells is intrinsic. To confirm this notion, we then compared CD1d expression levels on the surfaces of immune cells and the potential of APCs to present α-GalCer to NKT cells between NZB and NZW mice. Fig. 3C shows that there were no differences in CD1d expression levels on CD3+ T cells, B220+ B cells, CD11b+ macrophages, or CD11c+ dendritic cells between NZB and NZW mice. To compare the potential of APCs to present α-GalCer, the APC-rich cell suspension, obtained by depletion of T cells, NK cells, and NKT cells using complement-dependent lysis of spleen cells with Abs to Thy1.2, CD4, CD8, asialoGM1, were cocultured with the cell suspension of an NKT hybridoma cell line, N38–3C3 (34), in the presence of varying concentrations of α-GalCer. As shown in Fig. 3D, APCs from both NZW and NZB mice were able to present α-GalCer to the NKT hybridoma cells, as estimated by IL-2 levels produced by the hybridoma cells.

FIGURE 4. Locus controlling cytokine-producing potential of NKT cells. A, QTL scan of chromosome 11 for the amount of IL-4 produced in vitro by αGalCer-stimulated spleen cells in 205 (NZB × NZW)F1 × NZW backcross mice. The LOD score curve is shown on the right with the scale on the bottom. Map positions of microsatellite markers are arranged from the centromere to the telomere on the left of the chromosome line. B, Correlation of the amounts of IL-4 and IFN-γ produced by α-GalCer-stimulated spleen cells with NZW-derived D11Mit14 genotype in 205 (NZB × NZW)F1 × NZW and 83 (NZB × NZW)F1 × NZW backcross mice. Spleen cells from 2-mo-old backcross mice were cultured for 2 days in the presence of α-GalCer (30 ng/ml), and the concentrations of IL-4 and IFN-γ in the culture supernatants were measured by ELISA. The results of individual mice are shown as dots, and the mean ± SEM is shown on the right side. W/W, B/W, and B/B represent NZW/NZW, NZB/NZW, and NZB/NZB genotypes, respectively, for the D11Mit14 microsatellite marker. ***p < 0.001; **p < 0.01; and *p < 0.05.

FIGURE 5. Correlation of high serum levels of total IgG, IgG anti-DNA Abs, and severity of proteinuria with the NZW-derived D11Mit14 genotype and H-2 haplotype in 262 (NZB × NZW)F1 × NZW backcross mice. Backcross mice were divided into two or four groups according to D11Mit14 genotype, H-2 haplotype, or the combination of both, and disease phenotypes were compared at 8 mo of age among each group. Data represent the means ± SEM. ***p < 0.001; **p < 0.01; and *p < 0.05.
Locus controlling cytokine production by NKT cells

To identify the genetic locus controlling in vitro cytokine production by α-GalCer-stimulated NKT cells, a genome-wide QTL analysis was performed using 108 microsatellite markers in 205 (NZB × NZW)F1 × NZW backcross mice at 2 mo of age. As shown in Fig. 4A, we identified one QTL for the high IL-4-producing phenotype tightly linked to the microsatellite marker D11Mit14 (57 cM from the centromere: map position in Mouse Genome Informatics database) on NZW chromosome 11 with significant linkage (LOD of 3.6). To further estimate the significance level of this linkage, a permutation test was performed. The result showed that the likelihood ratio statistic value was 17.2, a figure higher than the critical value of significant linkage (12.6).

In these backcross mice, the group of progeny with the NZW/NZW (W/W) genotype for D11Mit14 showed significantly higher levels of not only IL-4 but also IFN-γ than did the progeny with the NZB/NZW (B/W) genotype for D11Mit14 (Fig. 4B), suggesting that the D11Mit14-linked NZW locus is also involved in the up-regulation of IFN-γ-producing potential of NKT cells. This was supported by studies using 83 (NZB × NZW)F1 × NZB backcross mice, in which the group of progeny with the NZB/NZW (B/W) genotype for D11Mit14 showed significantly higher levels of not only IL-4 but also IFN-γ than did the progeny with the NZB/NZB (B/B) genotype for D11Mit14 (Fig. 4B).

Correlation between the genotype for D11Mit14 and SLE phenotypes

To determine whether the D11Mit14-linked NZW locus contributes to SLE susceptibility, the correlation between D11Mit14 genotype and serum levels of total IgG, IgG anti-DNA Abs, and the severity of proteinuria was examined using 262 8-mo-old (NZB × NZW)F1 × NZB backcross mice. As shown in Fig. 5, the progeny with the NZB/NZW (B/W) genotype for D11Mit14 showed significantly higher serum levels of total IgG and IgG anti-DNA Abs and developed more severe proteinuria than did the progeny with the NZB/NZB (B/B) genotype.

As we reported previously (36–38), the H-2 haplotype is an important genetic element for severe SLE in (NZB × NZW)F1.
mice. This was evident in studies of backcross progeny, in which H-2 heterozygous progeny (B/W:H-2^dcs) showed much more severe SLE phenotypes than did the H-2 homozygotes (B/B:H-2^dbs). To assess interactions of the D11Mit14-linked locus and the H-2 haplotype, we divided backcross mice into four groups according to D11Mit14 genotype and H-2 haplotype and compared the culture severity. The ANOVA suggested that the D11Mit14-linked locus and H-2^dbs heterozygosity appear to play roles in exacerbation of (NZB × NZW)F1 disease in an additive manner (Fig. 5).

Effect of α-GalCer-stimulated NKT cells on Ig synthesis

To investigate the mechanism that underlies the correlation between the D11Mit14 genotype and serum autoantibody levels in studies of the backcross progeny, we examined the effect of α-GalCer-stimulated NKT cells on Ig synthesis by B cells. Spleen cells from 2- and 6-mo-old NZB, NZW, and (NZB × NZW)F1 mice were cultured in vitro in the presence or absence of α-GalCer, and Ig levels in the culture supernatants were examined. As shown in Fig. 6A, the addition of α-GalCer to the cell cultures significantly enhanced IgM, IgG, and IgM anti-DNA Ab synthesis in NZW and (NZB × NZW)F1 mice, but not in NZB mice. A small but significant amount of IgG anti-DNA Abs was detected in spleen cell cultures of 6-mo-old (NZB × NZW)F1 mice; however, α-GalCer-stimulation showed no enhancing effect.

To clarify that such up-regulation of Ig synthesis is due to the activation of NKT cells, we obtained T cell-depleted spleen cells (NKT + B) by negative sorting of α-GalCer-CD11c^-CD3^- T cells and NKT cell-depleted spleen cells (T + B) by negative sorting of α-GalCer-CD11c^-CD3^- NKT cells from 6-mo-old (NZB × NZW)F1 mice, and cultured them in the absence or presence of either α-GalCer or immobilized anti-CD3 mAb. The production of IgM, IgG, and IgM anti-DNA Abs was up-regulated in cultures of NKT + B cells in the presence of α-GalCer or anti-CD3 mAb, but not in cultures of T + B cells in the presence of anti-CD3 mAb (Fig. 6B). The amount of IgG anti-DNA Abs was below the detectable level in this culture system (data not shown). These findings may support the view that the α-GalCer-stimulated paradigm up-regulation of Ig synthesis by B cells is not due to the effect of trans-activated T cells.

Discussion

In the present study, we found that although NZB and NZW mice have normal frequencies of invariant TCR-bearing NKT cells, their potentials for cytokine production were remarkably different. Upon stimulation with α-GalCer, NKT cells from NZB mice were defective in producing both IL-4 and IFN-γ, while such potential of NKT cells in NZW mice was highly up-regulated. The cytokine production level of F1 hybrid progeny of NZB and NZW was intermediate. As the mode of cytokine production differed between NKT cells and T cells, these two types of cells appear to be under the control of separate mechanisms.

Because the aberrant cytokine-producing potential of NKT cells in New Zealand strains of mice was evident in young mice before the onset of immunological abnormalities, certain genetic mechanisms were thought to underlie this aberration. Genome-wide QTL analysis mapped one significant locus tightly linked to a microsatellite marker, D11Mit14 (57 cM from the centromere), on telomeric chromosome 11 for controlling IL-4 synthesis by α-GalCer-stimulated spleen cells. The significance of this locus, provisionally designated Ntm1 (NKT cell modifier 1) in this paper, was supported by findings in progeny studies, indicating that the D11Mit14 genotype of the NZW strain correlated significantly with the elevated potential of α-GalCer-stimulated spleen cells to produce IL-4. Although QTL analysis for IFN-γ production did not show a significant linkage, the association studies suggested that the D11Mit14 is also involved in the control of IFN-γ production by NKT cells. The significance levels of the difference in IFN-γ production between W/W and B/W and between B/B and B/W for the D11Mit14 genotype in the backcross progeny were lower than the levels in IL-4 production in backcross studies. Thus, it is conceivable that the genetic control of IFN-γ production by α-GalCer-stimulated NKT cells is more complex than that of IL-4, involving an additional locus or loci. Further genetic analysis is required to solve this issue.

Matsuki et al. (39) reported that the number and function of invariant TCR-bearing NKT cells in type I diabetes-prone NOD mice are regulated by several genes, including a gene (or genes) located in the Idd4 interval on chromosome 11. These authors showed that congenic B6 mice bearing NOD-Idd4 had reduced numbers of hepatic invariant NKT cells, resulting in a reduced cytokine response following in vivo activation with α-GalCer (39). This Idd4 interval covers a broad segment, including the D11Mit14-linked Ntm1 locus. At present, the genes in the Idd4 and Ntm1 loci are as yet unidentified. In the vicinity of D11Mit14, there is a gene for G-CSF (57 cM from the centromere). It is known that NKT cells express receptors for G-CSF (40). Crough et al. (41) reported that administration of G-CSF results in polarization of NKT cells toward a Th2 cytokine-secreting phenotype following α-GalCer stimulation. Thus, G-CSF may have the potential to influence cytokine production by NKT cells. However, α-GalCer-stimulated NZW and NZB NKT cells did not polarize to either Th1 or Th2 cytokine-producing phenotypes in the present study. Furthermore, because the aberrant cytokine-producing phenotypes in NZB and NZW NKT cells are intrinsic to NKT cells per se, the G-CSF gene itself may not be the gene under investigation.

Since Th1/Th2 balance is critical in immune regulation, it is important to determine its correlation to the underlying mechanism controlling cytokine production by NKT cells. In the regulation of CD4^+ Th1/Th2 cells, Gorham et al. (42) mapped a locus, Tpm1 (T cell phenotype modifier 1), to the middle region on chromosome 11, using crosses of Th1-polarized B10.D2 and Th2-polarized BALB/c strains. This interval contains several potent candidate genes, such as genes for IL-4 and IFN regulatory factor-1, both of which are mapped at 29 cM from the centromere. Another molecule possibly involved in the regulation of Th1/Th2 balance of CD4^+ T cells are T cell membrane proteins encoded by Tim (T cell Ig mucine) family genes (43, 44). Th1 and Th2 cells express Tim-3 and Tim-1, respectively, and the interaction of Tim proteins and their ligands is suggested to be involved in Th1/Th2 balance. Tim genes are located 5–10 cM upstream from Tpm1 (42) on chromosome 11. Thus, both Tpm1 and Tim genes are not linked to the D11Mit14-linked locus Ntm1 for NKT cells. Together with our present findings that the mode of cytokine production differs between T cells and NKT cells in New Zealand mice, it seems likely that separate genetic mechanisms are involved in the control of their cytokine-producing potential between T cells and NKT cells. In support of this view, we found recently that the in vitro IL-4-producing potential of naive T cells is tightly linked to the polymorphic IL-4Rα-chain gene on chromosome 7 (45).

Zeng et al. (31) reported that activation of NKT cells following in vivo administration of α-GalCer in (NZB × NZW)F1 mice induces Th1-type immune responses that exacerbate SLE in these mice. Studies by Foret et al. (32) indicated that the development of SLE in (NZB × NZW)F1 mice was associated with an expansion and activation of NKT cells, and that activation of NKT cells in these mice in vivo or in vitro with α-GalCer revealed a marked increase in their potential to contribute to the production of
IFN-γ with advancing age and disease progression. These findings may support the notion that NKT cells in (NZB × NZW)F₁ mice play a promoting, rather than a suppressive, role in the pathogenesis of SLE. The results of our present study are consistent with this notion; however, several points require discussion. First, NKT cells from (NZB × NZW)F₁ and NZB mice showed a significant age-associated increase; however, our results showed that they did not undergo proportional expansion. This may be because we only examined the mice at 2 and 8 mo of age. Forester et al. (32) indicated that in the spleens from aged (NZB × NZW)F₁ mice, because of the remarkable hypercellularity that developed in this organ during the progression of SLE, expansion of the total number of NKT cells was not reflected as proportional expansion. These authors showed evidence that both the percentage and the absolute number of NKT cells are increased in the spleen of mice at 14 wk of age, a time when these mice have not yet developed significant disease phenotypes. Because invariant TCR-bearing NKT cells are self-reactive (46) and recognize lysosomal isogalactotrihexosylceramide (iGb3), a potent endogenous ligand for NKT cell activation (47), it seems feasible that NKT cells may expand in response to large amounts of such ligands released from apoptotic cells as inflammatory tissue injuries progress in a wide variety of tissues in older (NZB × NZW)F₁ mice. In contrast, NZB mice are prone to autoimmune hemolytic anemia and also spontaneously develop splenomegaly as animals age (33). In our present study, although they showed age-associated increases in the number of NKT cells in their enlarged spleens, these NKT cells, like those from young mice, produced minimal amounts of IL-4 and IFN-γ in response to α-GalCer. These findings also support our notion that NZW-type Ntm1 is defective, and thus different mechanisms are suggested to underlie the pathogenesis of autoimmune disease in NZB mice.

Second, our study shows evidence that α-GalCer-stimulated NKT cells in (NZB × NZW)F₁ mice produce considerable amounts of both IFN-γ and IL-4 even in mice at older ages, and the cytokine profile did not polarize to IFN-γ. As our research was limited to the in vitro responses of NKT cells to α-GalCer, further in vivo studies may be necessary to reach a definitive conclusion. To clarify further whether the aberrant NKT cell functions found in the in vitro studies are indeed involved in the pathogenesis of SLE in NZB/WF₁ mice, we are now on the way to establishing interval congenic New Zealand mouse strains carrying either the NZB-type or NZW-type allele for the D11Mit14 interval congenic New Zealand mouse strains carrying either the NZB-type or NZW-type allele for the D11Mit14 genotype. Taking advantage of these congenic strains, we intend to analyze the mechanism of the role of D11Mit14-linked alleles in aberrant NKT cell functions and the mechanisms of their contribution to SLE pathogenesis. Recently, NKT cells were found to produce additional cytokines such as IL-13 (48) and IL-21 (49). It will be interesting to see whether the D11Mit14-linked locus also regulates the production of these cytokines and whether their changes influence SLE phenotypes in (NZB × NZW)F₁ mice.

The NZW genotype for D11Mit14 correlates significantly with the serum levels of IgG, IgG anti-DNA Abs, and the severity of proteinuria in (NZB × NZW)F₁ × NZB backcross progeny, suggesting that the Ntm1 locus-mediated NKT cell function is linked to the (NZB × NZW)F₁ disease. The present in vitro studies demonstrated that, upon stimulation with α-GalCer, NKT cells from NZW and (NZB × NZW)F₁ mice, but not NZB mice, were able to promote polyclonal IgM, IgG, and IgM anti-DNA Ab production by B cells. These findings are consistent with the report of Forester et al. (32), indicating that anti-CD3 mAb-stimulated CD1d-restricted NKT cells derived from (NZB × NZW)F₁ mice are able to strongly augment B cell activation in vitro, as determined by polyclonal B cell proliferation and secretion of IgGs. Intriguingly, NKT cells from older (NZB × NZW)F₁ mice injected under the renal capsule in young F₁ mice enhanced the injury in renal glomeruli (30). The underlying mechanism for the up-regulated Ig synthesis mediated by activated NKT cells remains undetermined. Our preliminary studies showed that this event occurs in the absence of cognate interaction between B and NKT cells; however, addition of IL-4, IFN-γ, or a combination of both failed to augment Ig synthesis (data not shown), suggesting the involvement of other factors. In this respect, IL-21 is of particular importance, because this cytokine is produced by NKT cells and shows pleiotropic effects on the functions of several types of immune cells, including B cells (49).

Although NKT cells did promote polyclonal IgG syntheses in vitro by B cells from older (NZB × NZW)F₁ mice, they did not promote the IgG anti-DNA Ab synthesis. There are two possible explanations. First, self-reactive B cells in older (NZB × NZW)F₁ mice are already activated in vivo, and thus are unresponsive to additional activators. Our earlier findings may be relevant that while B cells from young (NZB × NZW)F₁ mice showed high proliferative responses to IL-2, B cells from older mice were unresponsive (50). Alternatively, NKT cells alone cannot provide signals for affinity maturation of anti-DNA B cells. We have reported previously that MHC class II haplotype heterozygosity, one derived from NZB (H-2d) and the other from NZW (H-2z), is a critical genetic element for severe SLE in (NZB × NZW)F₁ mice (36–38) through a mechanism that mediates IgM to IgG class switching and affinity selection of pathogenic self-reactive B cells (51, 52). Because this process requires both CD4+Th cells and self-Ag, MHC class II genes are thought to serve as a genetic candidate SLE modifier that acts to exacerbate disease in (NZB × NZW)F₁ mice (33). In contrast, NZB backcross progeny, suggesting the involvement of pathogenic IgG autoantibodies. Together with the findings in genetic studies that the effects of the NZW-type H-2 haplotype and Ntm1 are cooperative in the production of pathogenic IgG anti-DNA Abs in (NZB × NZW)F₁ mice, it is highly likely that the NZW-type Ntm1 contributes to the production of such pathogenic autoantibodies by stimulating polyclonal B cells, including anti-DNA B cells, to be susceptible to signals from MHC class II-restricted CD4+ anti-DNA Th cells. Thus, the NZW Ntm1 allele is considered to be a candidate SLE modifier that acts to exacerbate disease in (NZB × NZW)F₁ mice. Conversely, the defect in NZB Ntm1 may be related in part to the delayed onset of mild SLE in these mice. Indeed, there is evidence that the event of IgM to IgG class switching of anti-DNA Abs occurs much less in NZB than in (NZB × NZW)F₁ mice (33).

Accumulated evidence showed that NKT cells are a crossroad between innate immunity and adaptive immunity. Although autoimmune disease is Ag-driven, NKT cells may be involved in the modulation of disease phenotypes, in either promoting or protective roles. Mechanisms that lead to the difference in roles of NKT cells in autoimmune diseases are not clearly understood. Further studies on the genetic mechanisms of cytokine regulation in NKT cells may contribute to a more thorough understanding of their roles in a variety of autoimmune diseases.

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References


