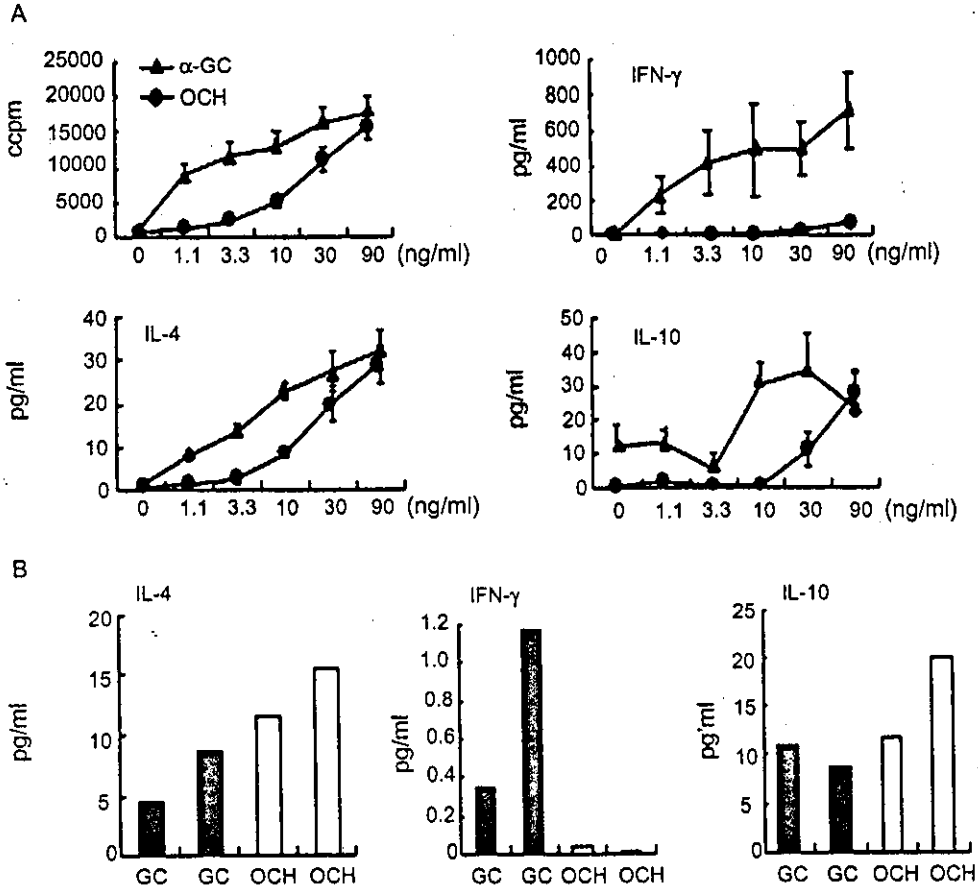

Fig. 1. Effects of OCH on cytokine production in NOD mice in vitro and in vivo. (A) Spleen cells were stimulated with various concentrations of glycolipid for 72 h. Incorporation of [^{3}H]-thymidine (1 μCi/well) for the final 16 h of the culture was examined (left top) and IFN-γ (right top), IL-4 (left bottom) or IL-10 (right bottom) contents in the supernatants were measured by ELISA. (B) Serum levels of IL-4, IFN-γ, IL-10 in NOD mice after intraperitoneal injection of OCH or α-GC were measured by ELISA. Data represent the individual mice and the peak level of each cytokine (2 h for IL-4, 24 h for IFN-γ and 6 h for IL-10) was displayed. Black bar: α-GC treated mice; white bar: OCH-treated mice.

16 weeks (Fig. 3). Massive infiltration of CD4 T cells, CD8 T cells, B cells into islets was observed in vehicle-treated NOD mice. Macrophages were also observed in vehicle-treated NOD mice. In contrast, OCH treatment inhibited infiltration of both T cells and B cells, and macrophages were barely detectable in OCH-treated mice. These results indicated that administration of OCH inhibited the development of diabetes and insulitis in NOD mice and the suppressive effect of insulitis is stronger than α-GC.

3.3. Administration of OCH promotes a Th2 response against autoantigens

To evaluate effects on immune responses against autoantigen, we measured GAD-specific IgG1 and IgG2a in the serum at the age of 24 weeks (Fig. 4, left and middle panels). The level of IgG2a was reduced both in OCH- and α-GC-treated mice compared to vehicle-treated mice. IgG1/IgG2a ratio significantly increases in OCH-treated mice. In α-GC-treated mice, IgG1/IgG2a ratio also tended to increase, even though the difference did not reach statistical significance. These results suggest that anti-GAD response in OCH-treated NOD mice shifted to Th2 response, and Th2 bias of autoantigen was more evident in OCH-treated mice compared to α-GC-treated mice.

3.4. OCH treatment increased IL-10 producing cells among islet-infiltrated leukocytes in NOD mice

Since anti-GAD response shifted to Th2 in OCH-treated mice, we next examined the numbers of IL-4 or IL-10 producing cells among the infiltrating leukocytes. IL-10 producing cells among CD45 positive leukocytes were significantly increased in OCH-treated mice compared to vehicle-treated NOD mice (Fig. 5). IL-4 producing cells were not significantly increased in OCH-treated mice. The frequency of either IL-4- or IL-10-producing cells among leukocytes in α-GC-treated mice were not significantly different from vehicle-treated mice. These results suggest that OCH-mediated

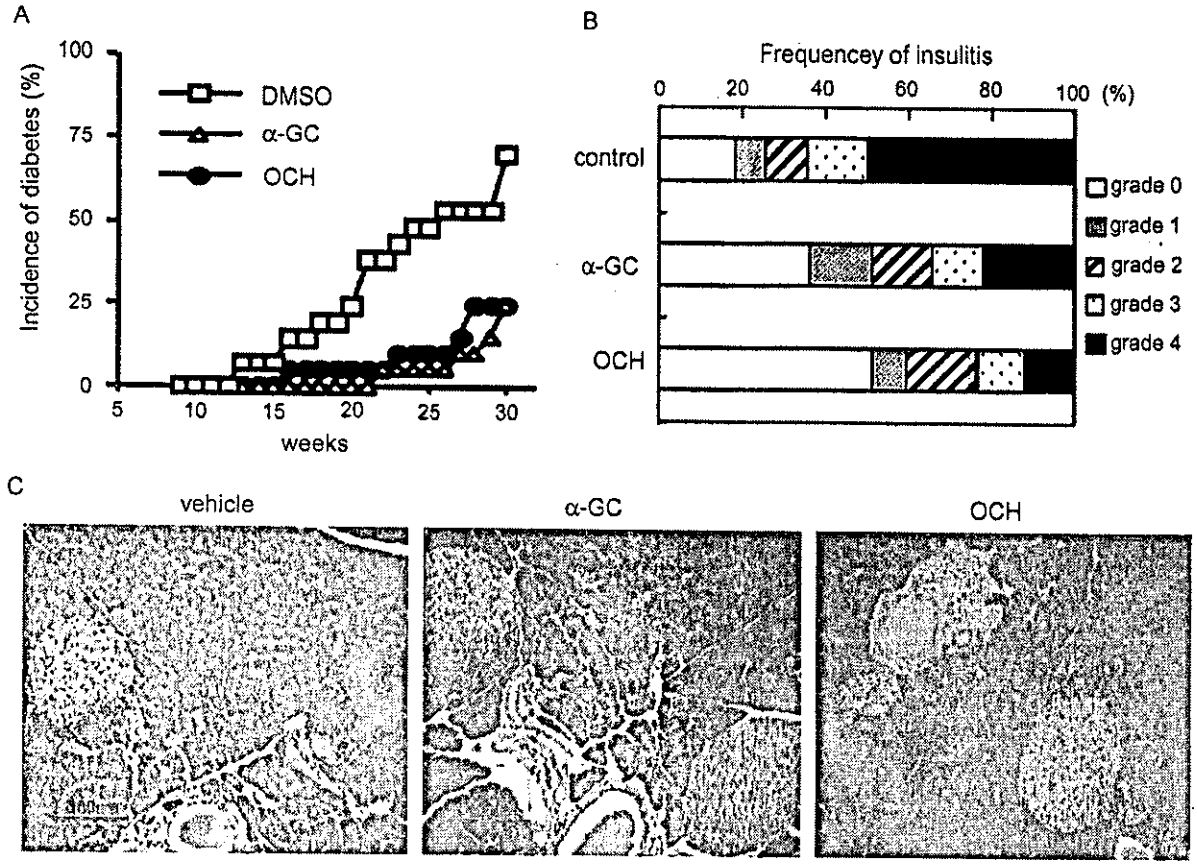

Fig. 2. Prevention of diabetes and insulitis in NOD mice by OCH treatment. (A) The effect of OCH on the incidence of diabetes. Female NOD mice were injected with OCH, α-GC or vehicle twice per week starting at 5 weeks of age ($n = 20$ per group). Diabetes was monitored by measurements of blood glucose levels. (B) Female NOD mice were treated with OCH, α-GC or vehicle twice per week starting at 5 weeks of age. Pancreata were microscopically evaluated for the degree of insulitis at 16 weeks of age. Data shown represent the mean of six animals in each group and more than 20 islets per mice were scored for grading as described in Section 2. The mean score of insulitis of vehicle-, α-GC-, or OCH-treated mice was 1.56, 1.24, 2.7 ($p < 0.001$; vehicle vs α-GC, $p < 0.001$; vehicle vs OCH, $p < 0.0081$; α-GC vs OCH). (C) Representative histological appearance of hematoxylin and eosin-stained islets from non-diabetic female NOD mice treated with vehicle, α-GC, or OCH used in the experiments shown in (B).

inhibition of insulitis is associated with increase of IL-10 producing cells.

4. Discussion

In this study, we found that OCH treatment prevented spontaneous autoimmune diabetes in NOD mice, a model of human type I diabetes, by inducing Th2 bias. OCH treatment strongly inhibited insulitis in NOD mice. The proportion of IL-10 producing cells among infiltrated leukocytes was increased in OCH-treated mice. Although the correlation between a defect in NKT cells and the susceptibility of diabetes in NOD mice is still debated [13–15], the putative involvement of NKT cells in the control of islet β-cell reactive T cells in NOD mice was suggested by the prevention of diabetes following an infusion of NKT cell-enriched thymocytes [16], and by the increase of NKT cells in Vα14-Jα281 transgenic NOD mice [17]. In these mice, protection from diabetes by NKT cells is associated with the induction of a Th2 response to islet autoantigens. Furthermore, α-GC treatment has been demonstrated to delay the onset and reduce the incidence of diabetes in NOD mice [18–21]. And the mechanism of protection has been reported to be associated with Th2 shift of autoantigen response, which is similar to that observed by increasing the number of NKT cells in NOD mice. The mechanism of OCH-mediated inhibition of insulitis was also associated with Th2 polarization of the autoantigen response and increase of IL-10 producing cells. The stronger suppression of insulitis by OCH treatment than α-GC treatment may not be surprising because α-GC stimulates NKT cells to produce IFN-γ as well as Th2 cytokines. IFN-γ enhances the expression of major histocompatibility complex class I and II molecules as well as several other proteins involved in antigen processing and presentation, and supports the homing of activated T cells into islets in NOD mice [27–29].

Stimulation of NKT cells by injection of OCH inhibited EAE and CIA [22,23]. On the other hand,

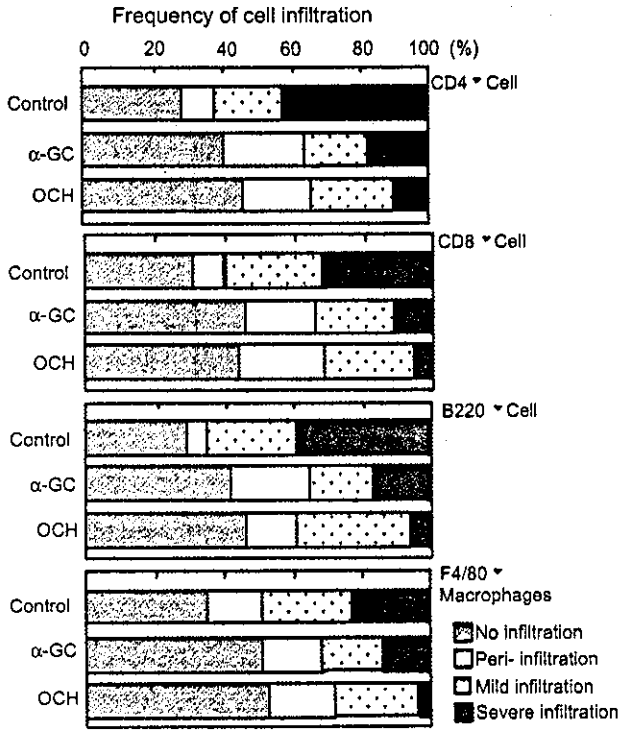

Fig. 3. Immunohistochemical analysis of cell composition of infiltrated cells into islets in NOD mice treated with OCH. Female NOD mice were treated with OCH, α-GC or vehicle twice per week starting at 5 weeks of age. At 16 weeks of age, pancreata were microscopically evaluated for the degree of infiltrated cells positive for CD4, CD8, B220 or F4/80 antigen as described in Section 2. Data shown represent the mean of six animals in each group and more than 20 islets per animal.

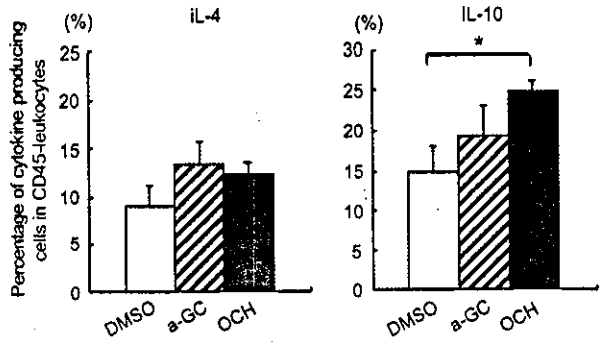

Fig. 5. Immunohistochemical analysis of IL-4 or IL-10 producing cells infiltrated into islets in NOD mice treated with OCH. Female NOD mice were injected with OCH, α-GC or vehicle twice per week starting at 5 weeks of age. Immunohistochemical examination was performed at 16 weeks of age. Data shown represent the mean of six animals in each group and more than 100 $CD45^+$ leukocytes per mice were evaluated. IL-4 positive or IL-10 positive cells were expressed as percentage of $CD45^+$ total leukocytes. $*p = 0.017$ vs control, by Dunnett's multiple comparison tests.

α-GC ameliorates or exacerbates EAE, depending on the strain of mouse and stage of disease tested [30–32] and to have only a marginal effect on CIA [23], probably because α-GC induces both Th1 and Th2 cytokines whereas OCH predominantly elicits a Th2 response. In this situation, treatment with OCH might be preferable to α-GC for Th1-mediated diseases. Although the insulitis was severe in α-GC-treated mice compared to OCH-treated mice, we confirmed that multiple injection of α-GC inhibited diabetes in NOD mice as well as OCH as previously reported [18–21]. There might be several reasons to explain that the effect of OCH was not different in the inhibition of overt diabetes. For example, it has been documented that no modification of the diabetes incidence occurs in both IFN-γ knockout and IFN-γ receptor knockout mice, as compared with littermate controls. Therefore α-GC induced IFN-γ has less effect to mask the protective effect of Th2 cytokines in NOD mice, which is different to other models such as EAE or CIA. The other possibility to explain that OCH and α-GC showed similar effect on the development of overt diabetes is that diabetes in NOD mice develops spontaneously and multiple injection of α-GC or OCH were continued for several months, which are different from other inducible autoimmune models. The adjuvant used for the induction of other models such as EAE and CIA might have some effect on the cytokine production by NKT cells. Considering that the bacterial infection has been shown to induce predominantly Th1 cytokines from NKT cells [33], injection of α-GC at the same time the injection of CFA including mycobacterium extract could enhance Th1 cytokine production from NKT cells

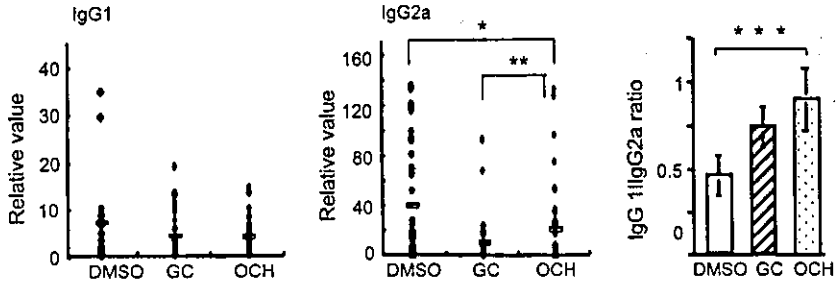

Fig. 4. GAD-specific antibody isotype levels in mice treated with OCH. Individual serum samples obtained from mice shown in Fig. 2 at 24 weeks were analyzed as in Section 2. IgG1:IgG2a ratios in OCH-, α-GC-, vehicle-treated mice are shown. Values are the mean and SEM ($n = 20$). $*p = 0.0327$, $***p = 0.0147$, OCH vs control, $**p = 0.0002$, α-GC vs control by Mann–Whitney U test.

enough for the mask of the inhibitory effect of Th2 cytokines. In contrast, in NOD mice which spontaneously develop diabetes, Th1 cytokine production from NKT cells for the initial treatment might not be so harmful because NKT cells have been reported to have the tendency to predominantly produce Th2 cytokines after repeated injections [34] since the glycolipid treatment was continued for several months.

It is still controversial whether the defects in NKT cells are causal for autoimmune disease or occur as a secondary consequence of the autoimmune process [13–15]. However, given the efficacy of glycolipid ligands such as OCH in the prevention of the development of diabetes in NOD mice, in addition to the suppression of EAE and CIA, stimulation of NKT cells with glycolipid seems to be an attractive strategy for the treatment of autoimmune diseases such as type I diabetes. The evolutionary conservation and the homogeneous ligand specificity of NKT cells allow us to apply a glycolipid ligand like OCH for the treatment of human disease without considering species barrier or genetic heterogeneity of humans.

Acknowledgements

This work was supported by the Pharmaceuticals and Medical Devices Agency (PMDA), Grant-in-Aid for Scientific Research (B) 14370169 from Japan Society for the Promotion of Science, Uehara Memorial Foundation, Kato Memorial Bioscience Foundation and Kanae Foundation for Life & Socio-Medical Science.

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Stimulation of Host NKT Cells by Synthetic Glycolipid Regulates Acute Graft-versus-Host Disease by Inducing Th2 Polarization of Donor T Cells[1]

Daigo Hashimoto,* Shoji Asakura,* Sachiko Miyake,† Takashi Yamamura,† Luc Van Kaer,‡ Chen Liu,§ Mitsune Tanimoto,* and Takanori Teshima[2]*¶

NKT cells are a unique immunoregulatory T cell population that produces large amounts of cytokines. We have investigated whether stimulation of host NKT cells could modulate acute graft-vs-host disease (GVHD) in mice. Injection of the synthetic NKT cell ligand α-galactosylceramide (α-GalCer) to recipient mice on day 0 following allogeneic bone marrow transplantation promoted Th2 polarization of donor T cells and a dramatic reduction of serum TNF-α, a critical mediator of GVHD. A single injection of α-GalCer to recipient mice significantly reduced morbidity and mortality of GVHD. However, the same treatment was unable to confer protection against GVHD in NKT cell-deficient CD1d knockout ($CD1d^{-/-}$) or $IL\text{-}4^{-/-}$ recipient mice or when $STAT6^{-/-}$ mice were used as donors, indicating the critical role of host NKT cells, host production of IL-4, and Th2 cytokine responses mediated by donor T cells on the protective effects of α-GalCer against GVHD. Thus, stimulation of host NKT cells through administration of NKT ligand can regulate acute GVHD by inducing Th2 polarization of donor T cells via STAT6-dependent mechanisms and might represent a novel strategy for prevention of acute GVHD. *The Journal of Immunology*, 2005, 174: 551–556.

Allogeneic hemopoietic stem cell transplantation (HSCT)[3] cures various hematologic malignant tumors, bone marrow (BM) failures, and congenital metabolic disorders. Emerging evidence suggests that allogeneic HSCT is also useful for treatment of other diseases, including solid tumors and autoimmune diseases (1, 2). However, graft-vs-host disease (GVHD) is a major obstacle that precludes wider application of allogeneic HSCT. The pathophysiology of acute GVHD is complex, involving 1) donor T cell responses to the host alloantigens expressed by host APCs activated by conditioning regimens (i.e., irradiation and/or chemotherapy), and 2) dysregulation of inflammatory cytokine cascades, leading to further T cell expansion and induction of cytotoxic T cell responses (3).

$CD4^+$ helper T cells can be divided into two distinct subpopulations: Th1 and Th2 cells (4). Th1 cells produce IFN-γ and IL-2, whereas Th2 cells produce IL-4, IL-5, and IL-13. Although the role of Th1 and Th2 cytokines in the pathophysiology of acute GVHD is complex and controversial (5–8), Th1 polarization of donor T cells predominantly plays a role in inducing the "cytokine storm" that is seen in several models of acute GVHD (3, 9), whereas Th2 polarization mostly suppresses inflammatory cascades and reduces acute GVHD (10–12). Many properties of dendritic cells (DCs), including the type of signal, the duration of activation, the ratio of DCs to T cells, and the DC subset that presents the Ag, influence the differentiation of naive $CD4^+$ T cells into Th1 or Th2 cells (13). The cytokines that are present during the initiation of the immune responses at the time when the TCR engages with MHC/peptide Ags are critically important for Th cell differentiation (14).

NKT cells are a distinct subset of lymphocytes characterized by expression of surface markers of NK cells together with a TCR. Although the NKT cell population exhibits considerable heterogeneity with regard to phenotypic characteristics and functions (15), the major subset of murine NKT cells expresses a semi-invariant TCR, Vα14-Jα18, in combination with a highly skewed set of Vβs, mainly Vβ8 (16). NKT cells can be activated via their TCR by glycolipid Ags presented by the nonpolymorphic MHC class I-like molecule CD1d expressed by APCs (17). Stimulation of NKT cells rapidly induces secretion of large amounts of IFN-γ and IL-4, thereby influencing the Th1/Th2 balance of conventional $CD4^+$ T cell responses (18). In particular, NKT cells are considered an important early source of IL-4 for the initiation of Th2 responses (19, 20), although these cells are not absolutely required for the induction of Th2 responses (21–23). NKT cells are absent in CD1d knockout ($CD1d^{-/-}$) mice because of defects in their thymic positive selection, which requires CD1d expression on hemopoietic cells, probably double-positive thymocytes (24, 25).

Considering the critical role of cytokines in the development of acute GVHD, we investigated the role of host NKT cells in an experimental model of GVHD, using synthetic NKT cell ligands,

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Received for publication August 6, 2004. Accepted for publication October 15, 2004.

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[1] This work was supported by research funds from the Ministry of Education, Culture, Sports, Science and Technology Grant 15591007 (to T.T.), by the Health and Labor Science Research Grants for Clinical Research for Evidence Based Medicine (to T.T.), by grants from the Ministry of Health, Labour, and Welfare of Japan (to T.Y.), by the Organization for Pharmaceutical Safety and Research (to T.Y.), and by a grant-in-aid for cancer research from the Fukuoka Cancer Society.

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[3] Abbreviations used in this paper: HSCT, hemopoietic stem cell transplantation; BM, bone marrow; GVHD, graft-vs-host disease; DC, dendritic cell; α-GalCer, α-galactosylceramide; BMT, bone marrow transplantation; TBI, total body irradiation; TCD, T cell depletion; LN, lymph node; WT, wild type.

0022-1767/05/$02.00

α-galactosylceramide (α-GalCer) (26), a glycolipid originally purified from a marine sponge, and its analog, OCH (27). Our findings indicate that stimulation of host NKT cells with NKT ligands can modulate acute GVHD.

Materials and Methods

Mice

Female C57BL/6 (B6, H-2^b) and BALB/c (H-2^d) mice were purchased from Charles River Japan. IL-$4^{-/-}$ B6 and STAT$6^{-/-}$ BALB/c mice were purchased from The Jackson Laboratory. CD1$d^{-/-}$ B6 mice were established by specific deletion of the CD1d1 gene segment (22). Mice, between 8 and 16 wk of age, were maintained in a specific pathogen-free environment and received normal chow and hyperchlorinated drinking water for the first 3 wk post-bone marrow transplantation (BMT). All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research Center.

Bone marrow transplantation

Mice were transplanted according to a standard protocol described previously (28). Briefly, B6 mice received lethal total body irradiation (TBI; x-ray), split into two doses separated by 6.5 h to minimize gastrointestinal toxicity. Recipient mice were injected with 5×10^6 BM cells plus 5×10^6 spleen cells from either syngeneic (B6) or allogeneic (BALB/c) donors. T cell depletion (TCD) of donor BM cells was performed using anti-CD90 MicroBeads and the AutoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. Donor cells were resuspended in 0.25 ml of HBSS (Invitrogen Life Technologies) and injected i.v. into recipients on day 0. Survival was monitored daily. The degree of systemic acute GVHD was assessed weekly by a scoring system incorporating five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity, as described (29).

Glycolipids

α-GalCer, (2S,3S,4R)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000), was synthesized and provided by Kirin Brewery Company (30). A homologue of α-GalCer, OCH, was selected from a panel of synthesized α-GalCer analogues by replacing the sugar moiety and/or truncating the aliphatic chains, because of its ability to stimulate enhanced IL-4 and reduced IFN-γ production by NKT cells, as previously described (27, 31). BMT recipient mice were injected i.p. with α-GalCer or OCH (100 μg/kg) immediately after BMT on day 0. Mice from the control groups received the diluent only.

Flow cytometric analysis

mAbs used were FITC- or PE-conjugated anti-mouse CD4, H-2K^b, and H-2K^d (BD Pharmingen). Cells were preincubated with 2.4G2 mAb (rat anti-mouse FcγR) for 10 min at 4°C to block nonspecific binding of labeled Abs, and then were incubated with the relevant mAbs for 15 min on ice. Finally, cells were washed twice with 0.2% BSA in PBS and fixed. After lysis of RBCs with FACS lysing solution (BD Pharmingen), cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). 7-Amino-actinomycin D (BD Pharmingen)-positive cells (i.e., dead cells) were excluded from the analysis. Fluorochrome-conjugated irrelevant IgG were used as negative controls. At least 5000 live events were acquired for analysis.

Cell cultures

Mesenteric lymph nodes (LNs) and spleens were removed from animals 6 days after BMT and four to six mesenteric LNs or spleens from each experimental group were combined. Numbers of cells were normalized for T cells and were cultured in complete DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.02 mM 2-ME, and 10 mM HEPES in wells of a 96-well flat-bottom plate, at a concentration of 5×10^4 T cells/well with 1×10^5 irradiated (20 Gy) peritoneal cells harvested from naive B6 (allogeneic) animals, or with 5 μg/ml plate-bound anti-CD3ε mAbs (BD Pharmingen) and 2 μg/ml anti-CD28 mAbs (BD Pharmingen). Forty-eight hours after the initiation of culture, supernatants were collected for the measurement of cytokine levels.

ELISA

ELISA was performed according to the manufacturer's protocols (R&D Systems) for measurement of IFN-γ, IL-4, and TNF-α levels, as described previously (32). Samples were obtained from culture supernatant and blood from retro-orbital plexus, diluted appropriately, and run in duplicate. Plates were read at 450 nm using a microplate reader (Bio-Rad). The sensitivity of the assays was 31.25 pg/ml for IFN-γ, 7.6 pg/ml for IL-4, and 23.4 pg/ml for TNF-α.

Histology

Formalin-preserved livers and small and large bowels were embedded in paraffin, cut into 5-μm-thick sections, and stained with H&E for histological examination. Slides were coded without reference to prior treatment and examined in a blinded fashion by a pathologist (C. Liu). A semiquantitative scoring system was used to assess the following abnormalities known to be associated with GVHD, as previously described (33): 0, normal; 0.5, focal and rare; 1.0, focal and mild; 2.0, diffuse and mild; 3.0, diffuse and moderate; and 4.0, diffuse and severe. Scores were added to provide a total score for each specimen. After scoring, the codes were broken and data were compiled. Pathological GVHD scores of intestine are the sum of scores for small bowel and colon.

Statistical analysis

Mann-Whitney *U* test was applied for the analysis of cytokine data and clinical scores. We used the Kaplan-Meier product limit method to obtain survival probability, and the log-rank test was applied for comparing survival curves. Differences in pathological scores between the α-GalCer-treated group and the diluent-treated group were examined by two-way ANOVA. We defined $p < 0.05$ as statistically significant.

Results

Administration of α-GalCer stimulates lethally irradiated mice to produce IFN-γ and IL-4

We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. B6 mice were given 13 Gy TBI and were injected i.p. with α-GalCer, OCH, or diluent 2 h after TBI. Six hours later, blood samples were obtained, and serum samples were prepared for measurement of IFN-γ and IL-4. TBI alone or BMT itself did not stimulate diluent-treated mice to produce IFN-γ or IL-4 (Fig. 1). Administration of α-GalCer increased serum levels of IFN-γ and IL-4, even in mice receiving TBI. However, serum levels of IFN-γ were much less in irradiated mice than in unirradiated mice. By contrast, the ability of irradiated mice to produce IL-4 to α-GalCer was maintained for 48 h after irradiation. Serum levels of IFN-γ and IL-4 in response to α-GalCer were not altered when irradiated wild-type (WT) mice were injected with 5×10^6 BM cells and 5×10^6 spleen cells isolated from allogeneic (BALB/c) donors. Furthermore, these cytokine responses were not observed when α-GalCer was injected into irradiated NKT cell-deficient CD1$d^{-/-}$ mice with or without BMT. These results suggest that host NKT cells that survive for at least 48 h after irradiation, rather than from infused donor cells, are critically involved in the production of these cytokines in response to glycolipids. Irradiation appears to impair the ability of mice to produce IFN-γ while preserving IL-4 production in response to α-GalCer. Similar cytokine profiles were observed when OCH was administered (data not shown).

Administration of α-GalCer to recipients polarizes donor T cells toward Th2 cytokine production after allogeneic BMT

Induction of GVHD fundamentally depends upon donor T cell responses to host alloantigens. We next evaluated the effect of glycolipid administration on donor T cell responses after allogeneic BMT in a well-characterized murine model of acute GVHD (BALB/c→ B6) directed against both MHC and multiple minor histocompatibility Ags. Lethally irradiated B6 mice were transplanted with 5×10^6 BM cells and 5×10^6 spleen cells from

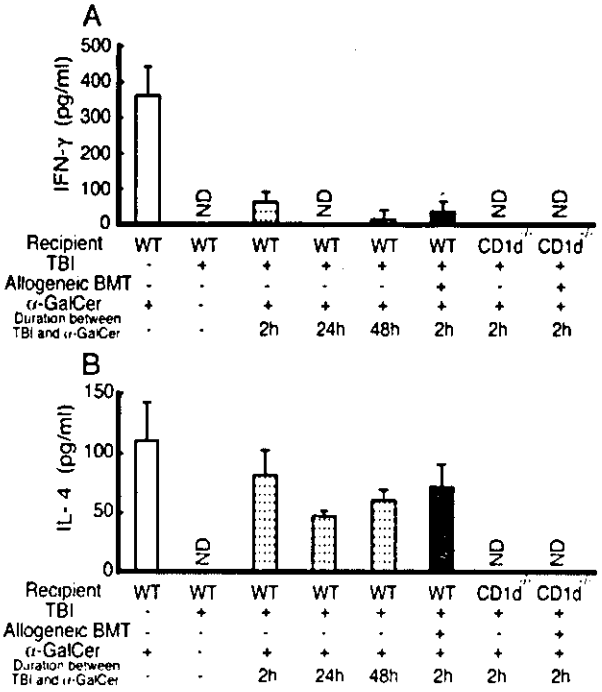

FIGURE 1. Cytokine responses to α-GalCer in lethally irradiated mice with or without BMT. WT and CD1d$^{-/-}$ B6 mice received 13 Gy TBI. Two, 24, or 48 h later, mice were injected i.p. with α-GalCer (100 μg/kg) or diluent. A cohort of animals were transplanted with allogeneic BM cells (5×10^6) and spleen cells (5×10^6) from WT BALB/c donors immediately after TBI, followed by injection of α-GalCer 2 h after TBI. Six hours after the administration of α-GalCer, serum samples were collected, and levels of IFN-γ (*A*) and IL-4 (*B*) were measured. α-GalCer-treated control mice without TBI (□), recipients of TBI plus α-GalCer (▨), and recipients of TBI, allogeneic BMT, and α-GalCer (■) are shown ($n = 3$ per group). Results represent one of three similar experiments and are shown as mean ± SD. *, $p < 0.05$ vs nonirradiated controls. ND, Not detected.

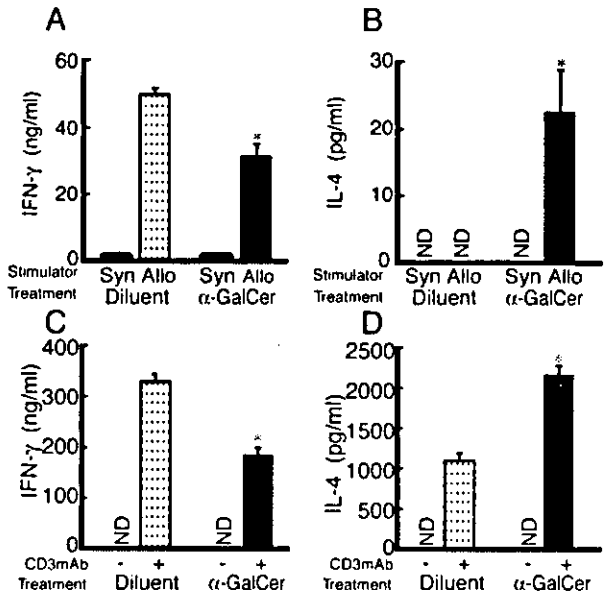

FIGURE 2. Administration of α-GalCer to recipients of allogeneic BMT polarizes donor T cells toward Th2 cytokine secretion. Lethally irradiated (13 Gy) B6 mice were transplanted with BM cells (5×10^6) and spleen cells (5×10^6) isolated from BALB/c mice, followed by injection of either α-GalCer or control diluent. Mesenteric LN cells obtained from diluent-treated recipients (□) and α-GalCer-treated recipients (■) 6 days after BMT were standardized for numbers of CD4$^+$ T cells as 5×10^4/well and were stimulated with 1×10^5/well of allogeneic or syngeneic peritoneal cells (*A* and *B*) or with CD3 (*C* and *D*). After 48 h, cytokine levels in the supernatant were measured by ELISA. Results shown are mean ± SD. *, $p < 0.05$ vs diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

either syngeneic (B6) or allogeneic (BALB/c) donors. Immediately after BMT, B6 recipients were injected i.p. with either α-GalCer or diluent. Six days after BMT, T cells isolated from mesenteric LN of recipient mice were cultured with irradiated B6 peritoneal cells or with anti-CD3ε mAbs and anti-CD28 mAbs for 48 h, and cytokine levels in the supernatant were determined. Flow cytometric analysis showed that >97% of LN T cells from both control recipients and α-GalCer-treated recipients were donor derived, as assessed by H-2^d vs H-2^b expression. T cells from α-GalCer-treated mice secreted significantly less IFN-γ, but more IL-4, in response to host alloantigens (Fig. 2, *A* and *B*) or to CD3 stimulation (Fig. 2, *C* and *D*) compared with those from controls. Similar results were obtained when T cells isolated from spleens were stimulated by anti-CD3ε and anti-CD28 mAbs. T cells from α-GalCer-treated mice secreted significantly less IFN-γ (18 ± 2 vs 164 ± 6 ng/ml), but more IL-4 (1022 ± 114 vs 356 ± 243 pg/ml), compared with controls. These results demonstrate that a single injection of α-GalCer to BMT recipients polarizes donor T cells toward Th2 responses after allogeneic BMT.

In α-GalCer-treated mice, serum levels of IFN-γ were dramatically reduced on day 6 compared with controls (Fig. 3*A*), and IL-4, which is usually hardly detectable in serum in this model, failed to be detected in the serum of mice of either group (data not shown). This impaired Th1 response of donor T cells was associated with a marked reduction of TNF-α levels in α-GalCer-treated mice (Fig. 3*B*).

Administration of α-GalCer or OCH to BMT recipients modulates acute GVHD

We next examined whether immune deviation mediated by administration of glycolipids can modulate acute GVHD. BMT was performed as above and α-GalCer was injected immediately after BMT on day 0. GVHD was severe in allogeneic controls, with 27% survival at day 50. A single injection of α-GalCer significantly improved survival to 86% ($p < 0.05$) (Fig. 4*A*). Allogeneic control mice developed significantly more severe clinical GVHD compared with syngeneic controls, as assessed by clinical GVHD scores (Fig. 4*B*). Clinical GVHD scores were significantly reduced in α-GalCer-treated recipients compared with allogeneic controls, but were greater than in syngeneic controls. Histological analysis showed that administration of α-GalCer significantly suppressed GVHD pathological scores in the intestine ($p < 0.05$). Analysis of donor cell engraftment at day 60 after BMT in spleens showed complete donor engraftment in α-GalCer-treated recipients (>99% H-2K^{d+}/H-2K^{b-} donor chimerism), ruling out rejection or mixed chimerism as a potential cause of GVHD suppression.

Similar protective effects against GVHD were observed in mice treated with OCH, further confirming the protective effects of NKT ligands (Fig. 4*C*). We performed BMT from B6 donors to BALB/c recipients to rule out strain artifacts. Again, a single injection of α-GalCer to BALB/c recipients reduced GVHD and significantly improved survival of animals (Fig. 4*D*).

Host NKT cells and host production of IL-4 are required for suppression of GVHD by α-GalCer

We examined the requirement of host NKT cells in this protective effect of α-GalCer, using NKT cell-deficient CD1d$^{-/-}$ mice as

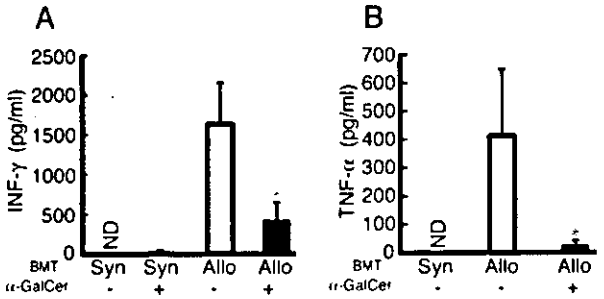

FIGURE 3. A single injection of α-GalCer to recipients of allogeneic BMT markedly reduces serum levels of IFN-γ and TNF-α. WT B6 mice were transplanted as in Fig. 2. Sera (n = 3–10/group) were obtained from diluent-treated (□) and α-GalCer-treated (■) recipients on day 6 after BMT, and serum levels of IFN-γ (*A*) and TNF-α (*B*) were determined. Results from three similar experiments are combined and shown as mean ± SD. *, $p < 0.05$ vs allogeneic, diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

BMT recipients. Lethally irradiated CD1d$^{-/-}$ mice were transplanted with BM cells and spleen cells from WT BALB/c donors, followed by administration of α-GalCer immediately after BMT on day 0. Protective effects of α-GalCer administration were not observed when CD1d$^{-/-}$ B6 mice were used as recipients, confirming the requirement for host NKT cells (Fig. 5*A*). We next examined the requirement of IL-4 production by host cells in this

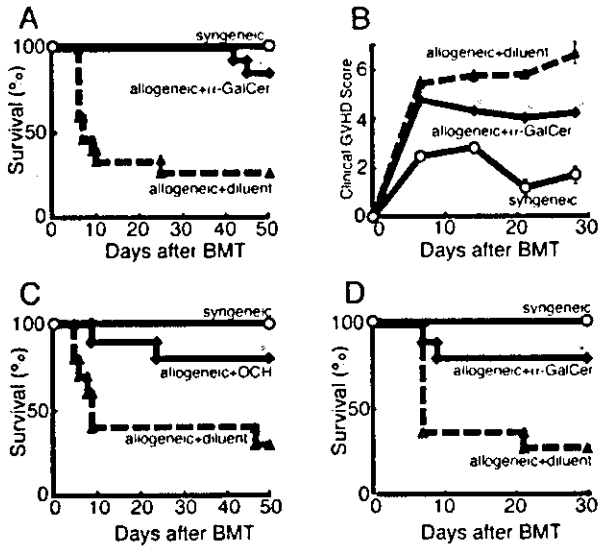

FIGURE 4. A single injection of NKT ligands to BMT recipients modulates acute GVHD. BMT was performed as in Fig. 2. *A*, Survival curves of syngeneic control group (○, solid line; n = 9); allogeneic, diluent-treated recipients (▲, dotted line; n = 15); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 14) are shown. Data from three similar experiments were combined. *B*, Clinical scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (◆, solid line) are shown as the mean ± SE. *C*, Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, OCH-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *D*, Lethally irradiated (9 Gy) BALB/c mice were transplanted from B6 donors. Survival curves of the syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *, $p < 0.05$ vs diluent-treated group.

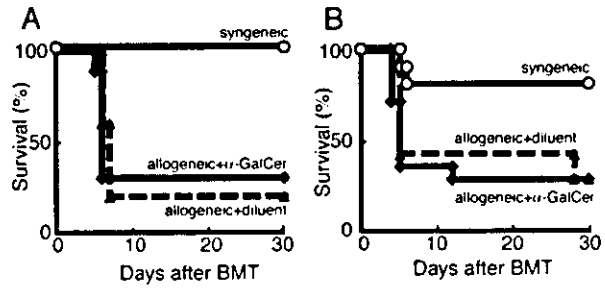

FIGURE 5. Host NKT cells and host IL-4 production are required for suppression of GVHD by α-GalCer. *A*, Lethally irradiated CD1d$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *B*, Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 11); allogeneic, diluent-treated recipients (▲, dotted line; n = 14); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 14) are shown. Data from three similar experiments were combined.

protective effect. Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted from WT BALB/c donors and administered α-GalCer as above. α-GalCer did not confer protection against GVHD in IL-4$^{-/-}$ recipients (Fig. 5*B*). Taken together, these results indicate that protective effects of α-GalCer are dependent upon host NKT cells and host production of IL-4.

STAT6 signaling in donor T cells is required for modulation of GVHD by α-GalCer

To determine whether IL-4-induced signaling in donor T cells is critical for the protective effect of glycolipids on GVHD, we used donor spleen cells that lack STAT6 and have impaired IL-4 responses (34, 35). Spleen cells from STAT6$^{-/-}$ BALB/c mice and TCD BM cells from WT BALB/c mice were transplanted after lethal TBI, followed by a single injection of α-GalCer. α-GalCer treatment failed to reduce morbidity and mortality of acute GVHD when STAT6$^{-/-}$ BALB/c donors were used (Fig. 6), demonstrating that STAT6 signaling in donor cells is critical for the protective effect of α-GalCer against GVHD.

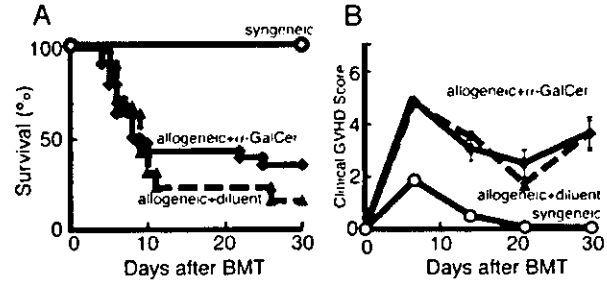

FIGURE 6. The protective effects of α-GalCer against GVHD are dependent upon the STAT6 pathway of donor T cells. Lethally irradiated B6 mice were transplanted with TCD-BM cells (4 × 10^6) from WT BALB/c mice and spleen cells (5 × 10^6) from STAT6$^{-/-}$ BALB/c mice. *A*, Survival curves of the syngeneic control group (○, solid line; n = 15); allogeneic, diluent-treated recipients (▲, dotted line; n = 25); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 25) are shown. Data from five similar experiments were combined. *B*, Clinical GVHD scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (◆, solid line) are shown as the mean ± SE.

Discussion

NKT cells are critically involved in the development and suppression of various autoimmune diseases. In experimental models, their regulatory mechanisms mostly depend on IL-4 production and subsequent inhibition of Th1 differentiation of autoreactive $CD4^+$ T cells (18). Previous studies have demonstrated that donor NKT cells regulate acute GVHD in an IL-4-dependent manner when administered together with donor inoculum (36). Considering these immunomodulating functions of NKT cells, we evaluated whether stimulation of host NKT cells could modulate GVHD in a mouse model of this disease.

Administration of α-GalCer stimulates NKT cells to produce both IFN-γ and IL-4 in naive mice, which can promote Th1 and Th2 immunity, respectively (18). We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. Surprisingly, irradiation of mice dramatically reduced IFN-γ production in response to α-GalCer, while preserving IL-4 production. This result may account for Th2, but not Th1, polarization of donor T cells by α-GalCer, even in conditions such as allogeneic BMT, which preferentially promotes Th1 polarization. Although mechanisms of selective suppression of IFN-γ production induced by irradiation need to be elucidated, irradiation may modulate the cytokine production profile of NKT cells or neighboring NK cells. Although OCH stimulates NKT cells to predominantly produce IL-4 compared with α-GalCer, resulting in potent Th2 responses (27, 31), both OCH and α-GalCer equally stimulate IL-4 production in irradiated mice and exert equivalent protection against acute GVHD.

Stimulation of host NKT cells by injecting α-GalCer or OCH polarized donor T cells toward Th2 cytokine secretion, resulting in marked reduction of serum IFN-γ levels after BMT. Th2 cytokine responses subsequently inhibited inflammatory cytokine cascades and reduced morbidity and mortality of acute GVHD, as previously described (10–12). Inflammatory cytokines have been shown to be important effector molecules of acute GVHD (37). α-GalCer treatment failed to confer protection against acute GVHD when $STAT6^{-/-}$ BALB/c donors were used, demonstrating that Th2 polarization via STAT6 signaling is critical for this protective effect of α-GalCer, although STAT6-independent Th2 induction has been reported (38, 39).

α-GalCer did not confer protection against GVHD in $CD1d^{-/-}$ or $IL\text{-}4^{-/-}$ recipients. Therefore, the protective effect of α-GalCer against GVHD is dependent upon host NKT cells and host production of IL-4. Sublethal total lymphoid irradiation enriches NKT cells in host lymphoid tissues, and these NKT cells induce Th2 polarization of conventional T cells by IL-4 production, resulting in reduced GVHD (40–42). These findings are consistent with our observation that IL-4 production is critical for the protective effects of NKT cells against acute GVHD. It should be noted, however, that systemic administration of IL-4 is either ineffective or toxic (6). Because the cytokine environment during the initial interaction between naive T cells and APCs is critically important for induction of Th1 or Th2 differentiation (14), local IL-4 production in the secondary lymphoid organs where donor T cells encounter host APCs might be necessary to cause effective Th1→Th2 immune deviation after allogeneic HSCT (43).

Current strategies for prophylaxis and treatment of GVHD primarily target depletion or suppression of donor T cells. These interventions suppress donor T cell activation and are associated with increased risk of infection and relapses of malignant diseases. Th1→Th2 deviation of donor T cells represents a promising strategy to reduce acute GVHD while preserving cytolytic cellular effector functions against tumors and infectious agents (33, 44–47). To achieve Th1→Th2 immune deviation of donor T cells, cytokines have been administered to either donors or recipients in animal models of GVHD. Donor treatment with cytokines such as IL-18 and G-CSF, and recipient treatment with IL-11, induces Th2 polarization of donor T cells and reduces acute GVHD (33, 44, 48). The present study reveals an alternative strategy to induce Th2 polarization of donor T cells by injecting NKT ligands into recipients to activate recipient NKT cells.

Prior studies (36, 40–42, 49) and the current study suggest that both donor and host NKT cells can regulate acute GVHD through their unique properties to secrete large amounts of cytokines and subsequent modulation of adaptive immunity. These studies reveal that there are several ways by which the NKT cell system can be exploited to suppress GVHD. First, administration of donor NKT cells expanded in vitro by repeated stimulation with glycolipid (50) can suppress GVHD (36). Second, total lymphoid irradiation enriches host NKT cells in lymphoid organs and thereby skews donor T cells toward Th2 cytokine production (40–42). Third, as shown here, administration of glycolipid to recipients stimulates host NKT cells to suppress GVHD. A recent phase I trial for patients with various solid tumors demonstrated that administration of α-GalCer was well tolerated with minimal side effects, which included temporal fever, headache, vomiting, chills, and malaise (51). Therefore, α-GalCer treatment may provide an effective and relatively safe option for preventing GVHD.

Cells belonging to the innate arm of the immune system, such as monocytes/macrophages, NKT cells, and NK cells, can produce large amounts of cytokines quickly upon stimulation. Innate immunity can thereby augment donor T cell responses to alloantigens in allogeneic HSCT (3). Our findings reveal a novel role for host NKT cells in regulating GVHD and indicate that stimulation of host innate immunity may serve as an effective adjunct to clinical regimens of GVHD prophylaxis.

Acknowledgments

We thank Kirin Brewery Company for providing synthetic α-GalCer and Keitaro Matsuo for statistical analysis.

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Cellular and Molecular Neurobiology, Vol. 24, No. 6, December 2004 (© 2004)

Gene Expression Profile Following Stable Expression of the Cellular Prion Protein

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Received April 20, 2004; accepted July 29, 2004

SUMMARY

1. The cellular prion protein (PrPC) is expressed widely in neural and nonneural tissues at the highest level in neurons in the central nervous system (CNS).

2. Recent studies indicated that transgenic mice with the cytoplasmic accumulation of PrPC exhibited extensive neurodegeneration in the cerebellum, although the underlying mechanism remains unknown. To identify the genes whose expression is controlled by overexpression of PrPC in human cells, we have established a stable PrPC-expressing HEK293 cell line designated P1 by the site-specific recombination technique.

3. Microarray analysis identified 33 genes expressed differentially between P1 and the parent PrPC-non-expressing cell line among 12,814 genes examined. They included 18 genes involved in neuronal and glial functions, 5 related to production of extracellular matrix proteins, and 2 located in the complement cascade.

4. Northern blot analysis verified marked upregulation in P1 of the brain-specific protein phosphatase 2A beta subunit (*PPP2R2B*), a causative gene of spinocerebellar ataxia 12, and the cerebellar degeneration-related autoantigen (*CDR34*) gene associated with development of paraneoplastic cerebellar degeneration.

5. These results indicate that accumulation of PrPC in the cell caused aberrant regulation of a battery of the genes important for specific neuronal function. This represents a possible mechanism underlying PrPC-mediated selective neurodegeneration.

KEY WORDS: *CDR34*; cellular prion protein; complement; extracellular matrix; microarray; neurodegeneration; *PPP2R2B*.

INTRODUCTION

Prion diseases are a group of neurodegenerative disorders affecting both animals and humans (Prusiner, 1998). The great majority of prion diseases are transmissible and characterized by intracerebral accumulation of an abnormal prion protein (PrPSc) that is identical in the amino acid sequence to the cellular isoform (PrPC) encoded by the *PRNP* gene. However, PrPSc differs biochemically from PrPC by its β-sheet-enriched structure, detergent insolubility, limited proteolysis by proteinase K, and a slower turnover rate (Prusiner, 1998). Previous studies suggest that the conversion of PrPC into PrPSc is mediated by a homotypic interaction between endogenous

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PrPC and incoming or de novo-generated PrPSc via an undefined posttranslational process (Prusiner, 1998). PrPC is postranscriptionally modified by the addition of two Asn-linked oligosaccharides that modulate the affinity of PrPC for a particular conformer of PrPSc (DeArmond *et al.*, 1997). PrPC is attached on the cell surface via a glycosylphosphatidylinositol (GPI) anchor. It is expressed widely in neural and nonneural tissues at the highest level in neurons in the central nervous system (CNS) (Bendheim *et al.*, 1992). Although the precise biological function of PrPC remains largely unknown, its location on the cell surface of neurons suggests a role of PrPC in neuritogenesis (Graner *et al.*, 2000), neuronal cell adhesion (Schmitt-Ulms *et al.*, 2001), and a receptor for neurotrophic factors (Chiarini *et al.*, 2002). Furthermore, an octarepeat region of PrPC with a copper-binding capacity is involved in the copper metabolism (Brown*et al.*, 1997). A previous study reported that a line of transgenic mice overexpressing PrPC showed necrotizing myopathy, demyelinating polyneuropathy, and focal vacuolation of the CNS (Westaway *et al.*, 1994). A more recent study showed that mice overexpressing a cytosolic form of PrPC exhibited extensive neurodegeneration in the cerebellum, although the mechanism underlying the selective neurodegeneration remains unknown (Ma *et al.*, 2002). These observations suggest that PrPC, when expressed aberrantly in the cell, becomes pathogenic.

Previously, several groups have established lines of PrPC-deficient (PrP $^{-/-}$) mice, designated Zür-I, Zür-II, Npu, Ngsk, Rikn, and Rcm0 by using different gene-targeting strategies (Büeler *et al.*, 1992; Kuwahara *et al.*, 1999; Manson *et al.*, 1994; Moore *et al.*, 1999; Rossi *et al.*, 2001; Sakaguchi *et al.*, 1996). All of these mice exhibited normal early development and complete protection against scrapie infection. These observations indicate that PrPC is dispensable for embryonic development, but is essential for inducing prion diseases. Zür-I mice showed an impairment of $GABA_A$ receptor-mediated fast inhibition and long-term potentiation in hippocampal CA1 pyramidal neurons, suggesting that PrPC is necessary for synaptic function (Collinge *et al.*, 1994). Both Zür-I and Npu mice showed an altered circadian activity rhythm and sleep pattern (Tobler *et al.*, 1996). Ngsk, Rikn, Rcm0, and Zür-II mice exhibited progressive cerebellar ataxia due to an extensive loss of Purkinje cells in the cerebellum, accompanied by an ectopic expression of downstream prion-like protein, Doppel (Dpl) (Moore *et al.*, 1999; Rossi *et al.*, 2001; Sakaguchi *et al.*, 1996). We previously showed that several genes located in the Ras and Rac signaling pathway, important for cell proliferation, differentiation, and survival, were aberrantly regulated in cultured fibroblasts isolated from Ngsk mice (Satoh *et al.*, 2000). However, very little is known about the genes whose expression is controlled by PrPC in human cells.

In the present study, we have established a stable human PrPC-expressing cell line designated P1 by the site-specific recombination method. This technique allows us to integrate the transgene into a single target site located in a transcriptionally active locus of the genome. It minimizes the opportunity for random integration of the transgene in the genome that potentially causes aberrant gene regulation, and excludes the detection of the genes regulated only transiently by the transfection procedure. To identify the genes whose expression is affected by accumulation of PrPC, the gene expression profile was compared on a 12,814 cDNA microarray between P1 and the parent PrPC-non-expressing cells.

MATERIALS AND METHODS

Establishment of a Stable PrPC-Expressing Cell Line

The human embryonic kidney HEK293 cells whose genome was modified for the Flp-In system were obtained from Invitrogen, Carlsbad, CA. They were named as F1 cells in the present study. F1 contains a single Flp recombination target (FRT) site targeted for the site-specific recombination, which is integrated in a transcriptionally active locus of the genome, where it stably expresses the *lacZ-Zeocin* fusion gene driven from the pFRT/*lacZ*eo plasmid under the control of SV40 early promoter. F1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (feeding medium) with inclusion of 100 μg/mL of Zeocin (Invitrogen).

The entire open reading frame (ORF) of the human *PRNP* gene (GenBank accession no. M13899) that encompasses both N-terminal and C-terminal signal sequences was amplified by polymerase chain reaction (PCR) from cDNA isolated from NTera2-derived human neurons (Satoh and Kuroda, 2000), using the sense and antisense primers (5'caccatggcgaaccttggctgctggat3' and 5'tccactatcaggaagatgagg aa3'). It was then cloned in a mammalian expression vector named pEF5/FRT/V5/D-TOPO which contains a C-terminal V5 tag sequence for detection of the recombinant protein (Invitrogen) (pEF5/FRT/PrPC-V5). The orientation and sequence of the cloned gene were verified by direct sequencing analysis. The sequence was identical to that of PrPC having five octapeptide repeats, Met at codon 129 and Glu at codon 219. Either the PrPC expression vector or the vector named pEF5/FRT/V5/GW-CAT which contains the chloramphenicol acetyltransferase (*CAT*) gene fused with a C-terminal V5 tag (Invitrogen) (pEF5/FRT/CAT-V5), together with the Flp recombinase expression vector pOG44 (Invitrogen), was transfected in F1 cells by Lipofectamine Plus reagent (Invitrogen). Stable cell lines were established after incubating the transfected cells for longer than 2 months in the feeding medium with inclusion of 100 μg/mL of Hygromycin B (Invitrogen) (the selection medium). The stable PrPC-expressing or CAT-expressing cell line was designated P1 or C1, respectively.

Transient Expression of PrPC in Human Neuronal Progenitor Cells in Culture

To identify the genes whose expression is controlled by transient overexpression of PrPC in human neural cells, the vector pEF5/FRT/PrPC-V5 or pEF5/FRT/CAT-V5 was transfected by Lipofectamine 2000 reagent (Invitrogen) in human neuronal progenitor (NP) cells isolated from the brain of a human fetus at 18.5-week gestation (BioWhittaker, Walkersville, MD). NP cells plated on a polyethyleneimine-coated surface were incubated in DMEM/F-12 medium containing an insulin–transferrin–selenium (ITS) supplement (Invitrogen, Carlsbad, CA), 20 ng/mL recombinant human epidermal growth factor (Higeta, Tokyo, Japan), 20 ng/mL recombinant human basic fibroblast growth factor (PeproTech EC, London, UK), and 10 ng/mL recombinant human leukemia inhibitory factor (Chemicon, Temecula, CA) (NP medium).

Microarray Analysis

P1, C1, and F1 cells were incubated for 5 days in the feeding medium without the addition of either Zeocin or Hygromycin B to exclude any potential effects of these antibiotics on gene expression. Then, total RNA was isolated from the cells by using TRIZOL reagent (Invitrogen) and RNeasy Mini Kit (Qiagen, Valencia, CA). Five micrograms of purified RNA was in vitro amplified once and the antisense RNA (cRNA) was processed for microarray analysis as described previously (Koike *et al.*, 2003). Two different arrays utilized in the present study include a 12,814 cDNA microarray (Agilent Technologies, Palo Alto, CA) and a 17,803 oligonucleotide microarray (Agilent Technologies). Both arrays contain the spots of sequence-known genes of various functional classes, including oncogenes, transcription factors, signal transducers, cell-cycle regulators, cytokines, growth factors and their receptors, apoptosis regulators, and housekeeping genes. The complete gene list is available on the World Wide Web (www.chem.agilent.com). For cDNA microarray analysis, cRNA isolated from P1 cells was labeled with a fluorescent dye Cy5, while cRNA of F1 cells was labeled with Cy3 by the reverse transcriptase reaction. The array was hybridized at 65°C for 17 h in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA. They were then scanned by the Agilent scanner (Agilent Technologies) and analyzed by using the Feature Extraction software (Agilent Technologies). The fluorescence intensities (FI) of individual spots were quantified following global normalization between Cy3 and Cy5 signals. The amount and quality of dye-labeled cDNA between two samples were verified by analyzing a scatter plot. The ratio of FI of Cy5 signal versus FI of Cy3 signal exceeding 3.0 was defined as significant upregulation, whereas the ratio smaller than 0.3 was considered as substantial downregulation.

Northern Blot Analysis

Three micrograms of total RNA was separated on a 1.5% agarose-6% formaldehyde gel, transferred onto nylon membranes, and immobilized by UV fixation. After prehybridization, the membranes were hybridized at 53°C overnight in the hybridization buffer containing a digoxigenin (DIG)-labeled DNA probe synthesized by PCR DIG probe synthesis kit (Roche Diagnosities, Mannheim, Germany) using the following sense and antisense primers: 5'catgtccgctgatgacctgaggat3' and 5'catagagggaac acagcttgctgc3' (the product size is 437 bp) for the protein phosphatase 2A β subunit (*PPP2R2B*) gene (M64930), 5'ggaagacgtggacttgctggaaga3' and 5'caggtcttccagtcagtca gtgtc3' (427 bp) for the cerebellar degeneration-related autoantigen (*CDR34*) gene (M16965), 5'tggcccgagatgcttaagtgtgac3' and 5'cgtcagcaagtactggctcttcac3' (396 bp) for the secreted frizzled-related protein 1 (*SFRP1*) gene (AI056087), 5'atgaggaagcaccctg agctggtgg3' and 5'gccccctctccaaccaaaaactcgca3' (456 bp) for the Doppel (*PRND*) gene (AI106918), and 5'ccatgttcgtcatgggtgtgaacca3' and 5'gccagtagaggcagggatgatgttc3' (251bp) for the glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) gene (J04038). After the probes were stripped by incubating the membranes in 0.1% SDS solution boiled for 10 min, they were processed for rehybridization with the DIG-labeled DNA probe specific for the *GAPD* gene for standardization of the amounts of RNA.

The specific reaction was visualized by exposing the membranes on Kodak X-OMAT AR X-ray films using the DIG chemiluminescence detection kit (Roche Diagnosities) as described previously (Satoh *et al.*, 2000; Satoh and Kuroda, 2000).

Western Blot Analysis

To prepare total protein extract, the cells were homogenized in RIPA lysis buffer composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and a cocktail of protease inhibitors (Roche Diagnosities), followed by centrifugation at 15,000 rpm for 20 min at room temperature (RT). The supernatant was collected for separation on a 12% SDS-PAGE gel as described previously (Satoh *et al.*, 1998). To identify the glycosylated PrPC isoforms, 40 μg of total cellular protein of P1 cells was deglycosylated by incubating the protein at 37°C for 3 h with 5,000 U peptide *N*-glycosidase F (PNGase F; New England BioLabs Beverly, MA) before separation on the gel. To identify the GPI-anchored PrPC isoforms, P1 cells were cultured overnight at 37°C in the serum-free DMEM, followed by incubation at 37°C for 3 h in the serum-free DMEM supplemented with 5 U/mL phosphatidylinositol-specific phospholipase C (PIPLC; Sigma, St. Louis, MO). Then, the culture supernatant was harvested and concentrated at an 1/30 volume by centrifugation on a Centricon-10 filter (Millipore, Bedford, MA).

After gel electrophoresis, the protein was transferred onto nitrocellulose membranes followed by immunolabeling at RT for 1 h with a mouse monoclonal antibody against V5 (1:10,000; Invitrogen). The membranes were then incubated at RT for 1 h with HRP-conjugated anti-mouse IgG (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA). After the antibodies were stripped by incubating the membranes at 50°C for 30 min in the stripping buffer composed of 62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100 mM 2-mercaptoethanol, they were processed for relabeling with a mouse monoclonal antibody against human PrPC reacting with an epitope located in the amino acid residues 95–110 (8G8, 1:500; Cayman Chemical, Ann Arbor, MI), a goat polyclonal antibody against the peptide mapping near the C-terminus of human PrPC (1:500; C-20, Santa Cruz Biotechnology), or with a goat polyclonal antibody against human heat shock protein HSP60, a housekeeping gene product (1:10,000; N-20, Santa Cruz Biotechnology) for standardization of the amounts of protein. They were followed by incubation with HRP-conjugated anti-mouse or goat IgG (1:5,000, Santa Cruz Biotechnology). The specific reaction was visualized by the Western blot detection system using a chemiluminescent substrate (Pierce, Rockford, IL).

Immunocytochemistry

Double-labeling immunocytochemistry was performed according to the methods described previously (Satoh *et al.*, 1998). In brief, the cells on cover glasses were either unfixed or fixed at 4°C for 10 min in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The latter was followed by incubation at RT for 10 min in phosphate-buffered saline (PBS) containing 0.5% Triton X-100. They were then incubated at RT for 15 min in PBS containing 10% goat serum and 10% human serum to block nonspecific binding of the antibodies. The cells were incubated at RT

for 30 min with 8G8 antibody (1:200), followed by incubation at RT for 30 min with rhodamine-conjugated anti-mouse IgG (1:80; ICN-Cappel, Aurora, OH). They were then incubated at RT for 5 min with 4',6'-diamidino-2-phenylindole (DAPI) (1:30,000; Molecular Probe, Eugene, OH). After several washes, cover glasses were mounted on slides with glycerol-polyvinyl alcohol, and examined on a Nikon ECLIPSE E800 universal microscope. Negative controls underwent all the steps except for exposure to the primary antibody.

RESULTS

Characterization of a Stable PrPC-Expressing Cell Line Designated P1

The human PrPC expression vector was transfected in F1 cells which have a defined FRT site in the genome for the site-specific recombination. A stable PrPC-expressing cell line was established by incubating the transfected cells in the selection medium for longer than 2 months and it was designated P1. For the control of transgene expression, another stable cell line expressing CAT was established by transfection of the CAT expression vector in F1 cells and it was designated C1. F1 expressed constitutively a weak 2.6-kb band of the cellular PrP mRNA, whereas P1 expressed an intense 1.2-kb transcript and several isoforms corresponding to the PrP transgene transcripts (Fig. 1(a), lanes 1,2). Both F1 and P1 did not express the Doppel mRNA (Fig. 1(c), lanes 1, 2). P1 expressed a single 36-kDa protein labeled

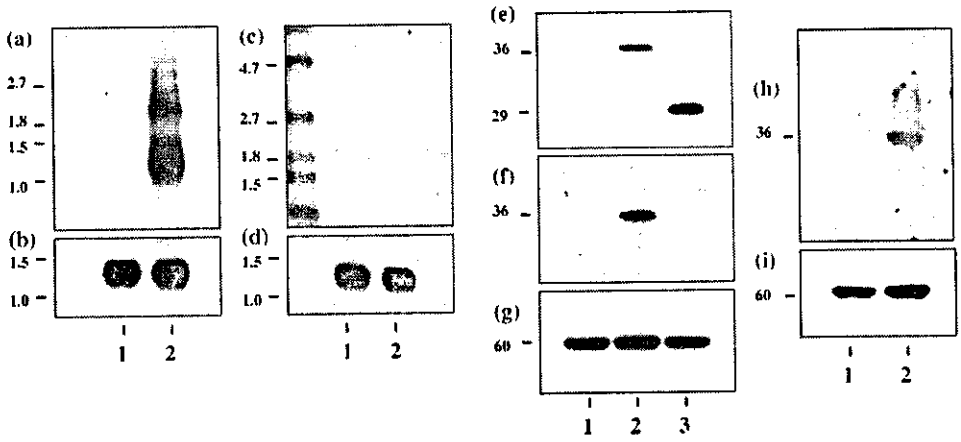

Fig. 1. Establishment of a stable PrPC-expressing cell line designated P1. A stable PrPC-expressing cell line designated P1 was established by transfection of the PrPC expression vector in HEK293 cells whose genome was modified for the Flp-In system (F1 cells). For the control of the transgene expression, another stable cell line expressing CAT was established and designated C1. They were processed for Northern blot analysis (3 μg of RNA per lane, panels a–d) and Western blot analysis (20 μg of protein per lane, panels e–i). The lanes 1–3 represent (1) F1, (2) P1, and (3) C1 cells. The panels a–i indicate (a) PrP mRNA; (b) glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) mRNA on the identical blot of (a); (c) Doppel (*PRND*) mRNA; (d) *GAPD* mRNA on the identical blot of (c); (e) V5; (f) PrPC (8G8) on the identical blot of (e); (g) HSP60 on the identical blot of (e); (h) PrPC (C20); and (i) HSP60 on the identical blot of (h). The RNA size marker is shown on the left in panels a–d. The molecular weight marker is indicated on the left in panels e–i.

with anti-V5 antibody and two different anti-human PrPC antibodies (8G8 and C20) (Fig. 1(e),(f), and (h), lane 2). Thus, this protein represents the recombinant human PrPC fused with a V5 tag in its C-terminus. On the other hand, C1 expressed a 29-kDa protein corresponding to the recombinant CAT-V5 fusion protein (Fig. 1(e), lane 3). The morphology and cell growth rate were not different among these cell lines.

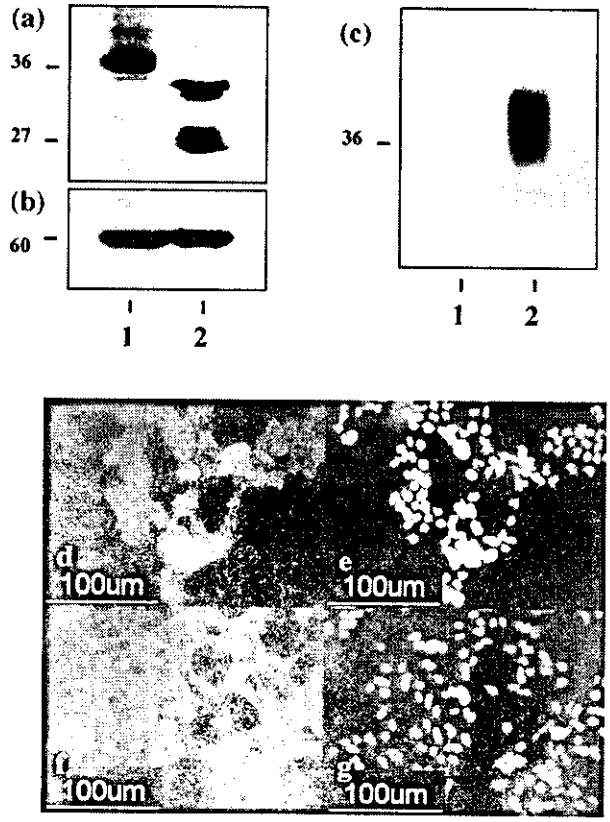

Fig. 2. Identification of PrPC in PI cells. To identify the glycosylated PrPC isoforms, 40 μg of total cellular protein of PI was deglycosylated by incubation with PNGase F at 37 C for 3 h, and it was then processed for Western blot analysis (panels a,b). The lanes 1, 2 represent (1) untreated and (2) PNGase F-treated protein. The panels a, b indicate (a) PrPC (8G8) and (b) HSP60 on the identical blot of (a). To identify the GPI-anchored PrPC isoforms, PI cells were incubated at 37 C for 3 h in the serum-free DMEM supplemented with PIPLC. Twenty microliters of the concentrated culture supernatant was processed for Western blot analysis using 8G8 (panel c). The lanes 1, 2 represent the culture supernatant of (1) untreated and (2) PIPLC-treated PI cells. The molecular weight marker is indicated on the left in panels a–c. To determine the subcellular location of PrPC, PI cells were processed for double-labeling immunocytochemistry. The panels d–g represent unfixed cells stained with (d) 8G8 or (e) DAPI, and 4% PFA-fixed, 0.1% Triton X-100-permeabilized cells stained with (f) 8G8 or (g) DAPI.

To identify the glycosylated PrPC isoforms, total cellular protein of P1 was incubated with PNGase F, and separated on the SDS-PAGE gel. After deglycosylation, two distinct bands of 33-kDa and 27-kDa labeled with 8G8 were identified in P1 (Fig. 2(a), lane 2). This indicates that the recombinant PrPC expressed in P1 was heavily glycosylated. To identify the GPI-anchored PrPC isoforms, the supernatant was collected from the culture of P1 before and after treatment of the cells with PIPLC. A broad 32- to 40-kDa band labeled with 8G8 (Fig. 2(c), lane 2) but not with V5 (not shown) was identified only in the culture supernatant of P1 cells after treatment with PIPLC. This suggests that a considerable amount of recombinant PrPC was processed physiologically to a GPI-anchored protein attached on the cell surface of P1 following the cleavage of the C-terminal V5 tag. By immunocytochemistry, P1 cells expressed an intense 8G8 immunoreactivity detectable not only on the surface (Fig. 2(d)) but also in the cytoplasm (Fig. 2(f)), while negative control cells showed no immunolabeling (not shown).

The Genes Differentially Expressed Between P1 and F1

A scatter plot exhibiting the comparison between Cy5 and Cy3 signals showed a well-confined linear pattern, indicating that the amount and quality of dye-labeled cDNA processed for hybridization in microarray analysis were quite similar between P1 and F1 samples (Fig. 3(a)). Among 12,814 genes on a cDNA array, 33 genes were found to be expressed differentially between P1 and F1 (Table I). Among them, 21 genes were upregulated, whereas 12 genes were downregulated in P1 cells (Table I). Because this array contains a spot derived from the 3′ non-coding sequence of the *PRNP* gene, PrP was not identified as one of upregulated genes in P1.

To verify the microarray data, three genes were selected for quantification by Northern blot analysis. P1 cells but neither F1 nor C1 expressed a 2.3-kb transcript of the *PPP2R2B* gene (Fig. 3(b), lane 2). Again, only P1 cells expressed a 1.5-kb transcript of the *CDR34* gene (Fig. 3(d), lane 2). In contrast, both F1 and C1 cells but not P1 expressed a 4.5-kb transcript of the *SFRP1* gene (Fig. 3(f), lanes 1,3). These observations indicate that the differential expression of *PPP2R2B*, *CDR34*, and *SFRP1* genes among these lines does not reflect an epiphenomenon due to overexpression of the transgenes or V5 tag, but represents PrPC overexpression-specific aberrant gene regulation.

Upregulation of Interferon-Responsive Genes Following Transient Overexpression of Exogenous Genes in Human Neuronal Progenitor Cells in Culture

To identify the genes whose expression is controlled by transient overexpression of PrPC in human neural cells, the PrPC expression vector or the CAT expression vector was transfected in NP cells by Lipofectamine 2000 reagent. Total cellular RNA was isolated at 48 h after transfection and then processed for microarray analysis. Upregulated expression of PrPC or CAT in the transfected cells was verified by Northern blot analysis and Western blot analysis (Supplementary Figure on the website). The gene expression profile was compared on a 17,803 oligonucleotide microarray between NP cells exposed to Lipofectamine 2000 reagent alone and

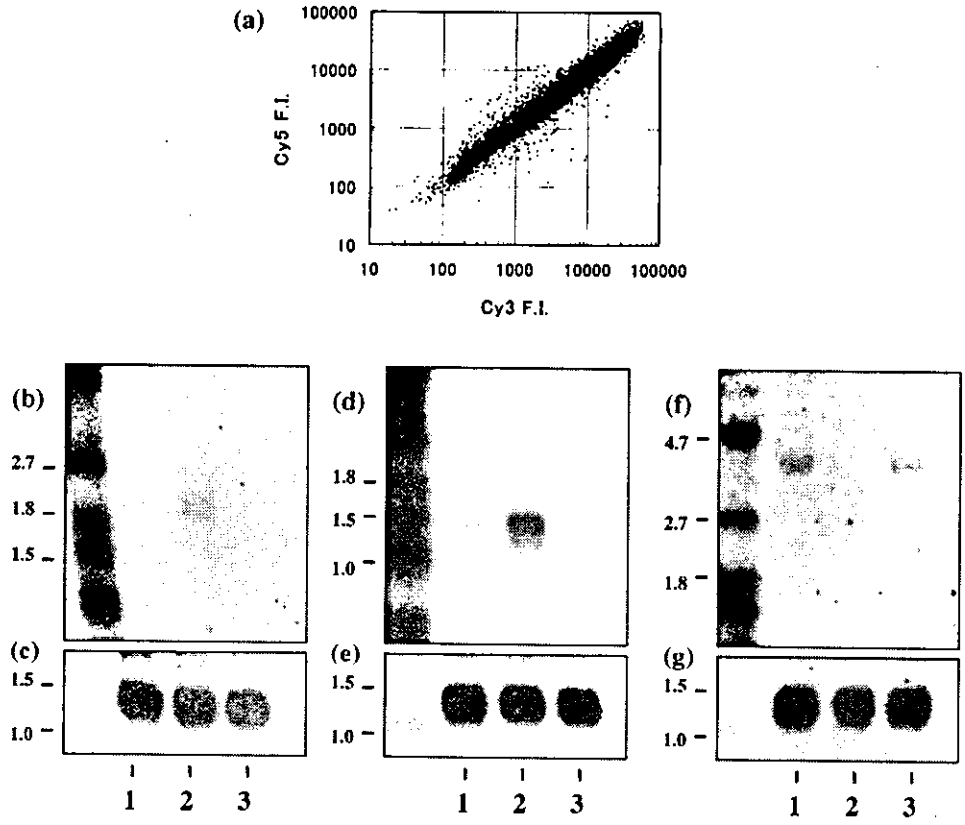

Fig. 3. Differential expression of *PPP2R2B*, *CDR34*, and *SFRP1* mRNA between PI and FL. The cDNA prepared from PI or FL cells labeled with Cy5 or Cy3, respectively, was processed for hybridization on a 12,814 cDNA microarray. The figure (a) represents a scatter plot exhibiting the comparison between the fluorescence intensity (FI) of Cy5 signals in the longitudinal axis and FI of Cy3 signals in the horizontal axis. To verify the microarray data, total cellular RNA of FL, PL, and CL cells was processed for Northern blot analysis (3 μg of RNA per lane, panels b–g). The lanes 1–3 represent (1) FL, (2) PL, and (3) CL cells. The panels b–g represent (b) protein phosphatase 2A beta subunit (*PPP2R2B*) mRNA; (c) *GAPD* mRNA on the identical blot of (b); (d) cerebellar degeneration-related autoantigen (*CDR34*) mRNA; (e) *GAPD* mRNA on the identical blot of (d); (f) secreted frizzled-related protein 1 (*SFRP1*) mRNA; and (g) *GAPD* mRNA on the identical blot of (f). The RNA size marker is shown on the left in panels b–g.

those transfected with the PrPC expression vector. One hundred genes were found to be upregulated in the PrPC expression vector-transfected NP cells. Surprisingly, 24 genes among them were categorized into known interferon (IFN)-responsive genes (Supplementary Table on the website). Northern blot analysis verified remarkable upregulation of IFN-induced protein with tetratricopeptide repeats 1 (*IFIT1*) and IFN-stimulated gene 15 (*G1P2*) in both PrPC expression vector-transfected and CAT expression vector-transfected NP cells but not in NP cells exposed to Lipofectamine 2000 reagent alone (Supplementary Figure on the website). These observations indicate that the global upregulation of IFN-responsive genes was not caused by treatment with the liposomal reagent, but induced by transient overexpression of exogenous genes in NP cells, which robustly activated the interferon system in