Ⅳ.　研究成果の刊行物・別刷

Chapter 17

Brain inflammation and psychogeriatric diseases

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BRAIN INFLAMMATION AND NEURONS

The long-held concept of the immune privilege of the brain has been revised significantly in the past 15 years. The blood–brain barrier (BBB) exists, but isolation from the periphery by the BBB is not as complete as it was thought to be and plays a limited role in the immune privilege. Recent evidence suggests that the brain parenchyma has unique mechanisms for regulating immune and inflammatory responses. For example, expression of CD200,[1] intercellular adhesion molecule (ICAM)-5/telencephalin[2] and transforming growth factor (TGF)-β[3] may contribute to the maintenance of the anti-inflammatory environment of the brain. Despite these mechanisms, low-grade inflammation occurs in association with brain lesions such as Alzheimer's disease,[4] cerebrovascular diseases and head trauma, with the full complexity of peripheral inflammatory responses. Studies on these non-immunological diseases have revealed that the brain has its own innate immune system and shares a variety of cytokines and other bioactive molecules with the peripheral immune system.

In the brain, microglia and astrocytes are thought to be the major cell populations that are engaged in the inflammatory responses. In a classic view, neurons are regarded as cells that perform only passive roles in inflammation. Neurons may become bystander victims that fuel the inflammatory processes by providing cell debris, which has to be removed by phagocytic cells and triggers further activation of these cells. However, there is a growing body of evidence that neurons not only respond to a number of inflammatory mediators but also produce many pro- and anti-inflammatory molecules.[5] These include complement proteins and inflammatory cytokines, as well as cyclo-oxygenase (COX)-2, prostaglandin E_2 receptors and inducible nitric oxide synthase, iNOS. Figure 17.1 illustrates the expression of COX-2 by hippocampal pyramidal neurons in an Alzheimer's disease patient, complicated with prolonged convulsion at the agonal stage. Interestingly, the literature indicates that some inflammatory molecules are involved in the modulation of neuronal functions such as neurotransmission.[5–7]

The physiological relevance of inflammatory molecules in neuronal activity leads to the hypothesis that mild or moderate inflammation, which does not visibly destroy brain tissue, can interfere with brain functions. It has been known that very strong systemic inflammation, as in

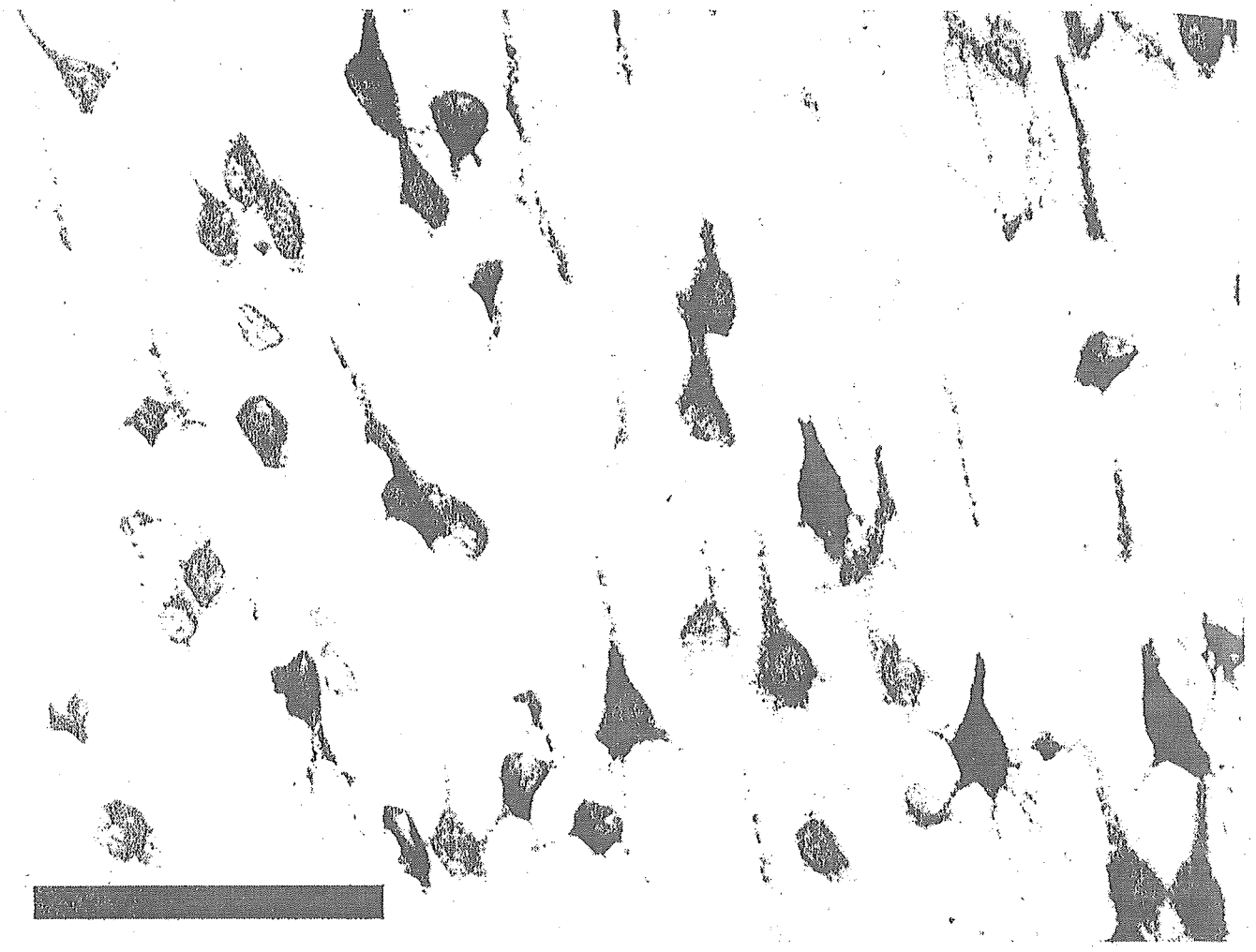

Figure 17.1 Cyclo-oxygenase-2 expression by hippocampal pyramidal neurons. An Alzheimer's disease case complicated with prolonged convulsion at the agonal stage. Scale bar, 100 μm

sepsis and toxic shock syndrome, are associated with central nervous system symptoms.[8,9] Clinical manifestations include delirium and other consciousness abnormalities, which are generally considered to be transient disorders. In these conditions, peripheral inflammation appears to disturb neural transmission in the absence of apparent encephalitic pathology. It has to be emphasized that, in elderly patients with such brain lesions as Alzheimer's disease or cerebrovascular diseases, even mild systemic inflammatory diseases frequently cause delirium.[6,10,11]

ACTIVATION OF VASCULAR CELLS BY SYSTEMIC INFLAMMATION

Vascular endothelial cells and perivascular cells are located at the interface between the peripheral blood and the brain parenchyma. These cells could therefore be involved in the transmission of inflammation from the periphery to the brain. The BBB may be effective in preventing large molecules such as complement and other inflammatory proteins from entering the brain. However, it may easily pass inflammatory signals through activation of these vascular cells.

We investigated activation of vascular endothelial cells and perivascular cells in the cerebral cortex of postmortem brain from patients with or without brain lesions. Many patients suffer from a variable degree of systemic inflammation at the agonal stage. In some cases, we used serum concentrations of an acute-phase reactant, C-reactive protein (CRP), as an index of systemic inflammation. Since the serum concentration of CRP at the agonal stage was available only in a limited number of patients, we estimated the degree of systemic inflammation by the intensity of immunohistochemical staining of the residual blood in brain tissue for CRP. In cases where we were able to obtain from the clinical records the serum CRP concentration on the day of or a day before death, intensity of CRP immunoreactivity in brain tissue showed a good correlation with serum CRP concentration.

Activation of vascular endothelial cells was investigated with three markers: ICAM-1, CD40 and COX-2. ICAM-1 is a cell adhesion molecule that belongs to the immunoglobulin superfamily. In the periphery, ICAM-1 expression plays a principal role in leukocyte adhesion to vascular endothelial cells and subsequent infiltration into tissues at the site of inflammation.[12] In the brain, reactive astrocytes and vascular endothelial cells express ICAM-1.[13] Figure 17.2a illustrates low expression of ICAM-1 by vascular endothelial cells in a control case without systemic inflammation. In cases without significant brain lesions, ICAM-1 immunoreactivity increased in parallel with the degree of systemic inflammation (Figure 17.2b). In neurological cases such as Alzheimer's disease, the correlation was less clear. A number of cases with brain lesions were high in vascular ICAM-1 expression even in the absence of systemic inflammation (Figure 17.2c).

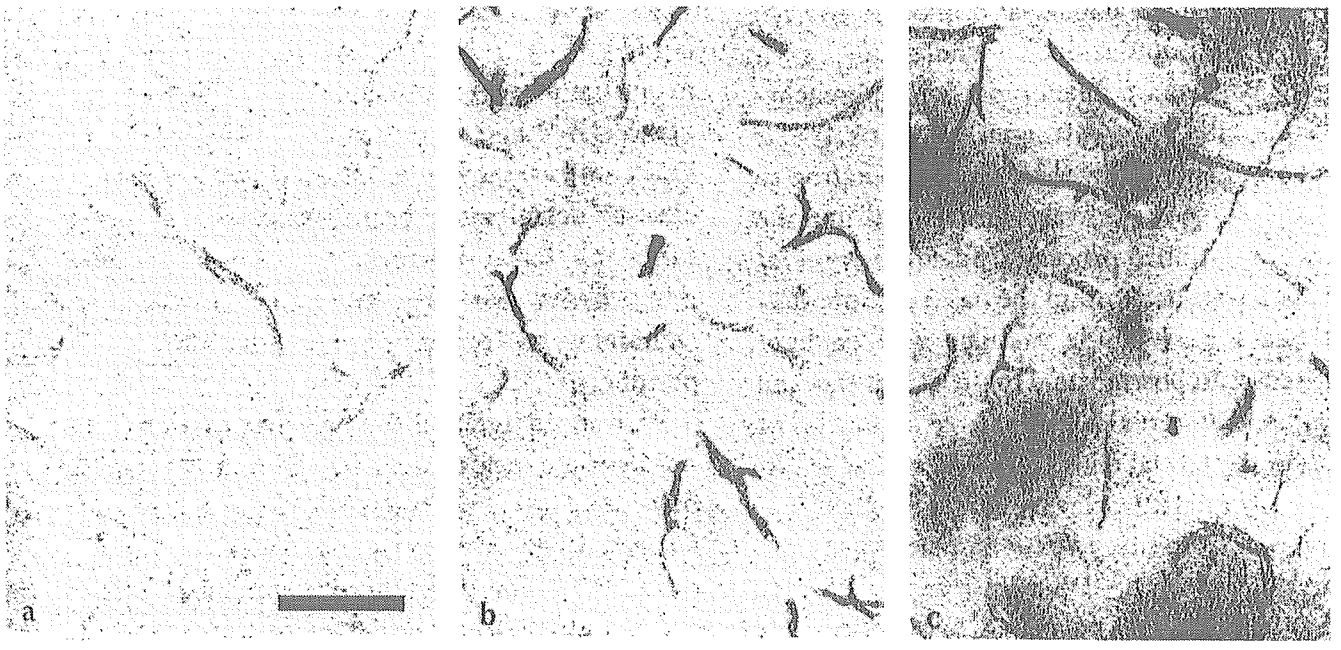

Figure 17.2 Intercellular adhesion molecule-1 (ICAM-1) expression by vascular endothelial cells in the temporal cortex. (a) A control case without systemic inflammation. Scale bar, 100 μm. a–c are at the same magnification. (b) A control case with strong systemic inflammation at the agonal stage. (c) A case with Alzheimer's disease without systemic inflammation. Patchy labeling of the brain parenchyma indicates ICAM-1 expression by reactive astrocytes around senile plaques. Note that vascular ICAM-1 expression is comparable with that in (b) even in the absence of systemic inflammation

The other two markers, CD40 and COX-2, showed similar changes. CD40 is a cell-surface molecule that is involved in immune and inflammatory processes and is overexpressed upon inflammation.[14,15] In the human brain, CD40 is expressed by reactive microglia and vascular endothelial cells.[16] COX is the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids. COX-2 is the inducible isoform of COX and is expressed by neurons and vascular endothelial cells in the brain. The intensity of vascular staining for CD40 and COX-2 generally paralleled the degree of systemic inflammation in cases without brain lesions, although a number of cases with brain lesions showed increased expression of CD40 and COX-2 by vascular endothelial cells in the absence of systemic inflammation.

Activation of perivascular cells was investigated with immunostaining for HLA-DR and CD68. Perivascular cells constitute, with meningeal macrophages, a monocyte-derived phagocytic population.[17] Therefore, perivascular cells share phenotypes with parenchymal microglia. We distinguished perivascular cells from microglia by their close association to collagen IV positive vasculature and by the absence of ramified processes. Since perivascular cells are embedded within the basal lamina, they look like a part of the vascular wall on light microscopy. In postmortem brain tissue, staining for HLA-DR revealed a variable intensity of perivascular cell labeling. The number of HLA-DR-positive perivascular cells in a given visual field varied from case to case. In many cases, the occurrence of HLA-DR-positive perivascular cells appeared to be increased in parallel with the degree of systemic inflammation. In cases with brain lesions, the relationship was less clear. It seemed that expression of HLA-DR by perivascular cells was increased by both systemic inflammation and brain lesions. The occurrence of CD68-positive perivascular cells showed similar changes; it was increased upon systemic inflammation. A number of neurological cases exhibited high CD68 expression by perivascular cells even in the absence of systemic inflammation.

INFLAMMATION AND DELIRIUM

Our results indicate that systemic inflammation activates vascular endothelial cells and perivascular cells in the brain. Brain parenchymal lesions also activate these vascular cells. In the brains of patients with neurodegenerative diseases, activated microglia and astrocytes are capable of producing a great variety of pro-inflammatory mediators,[4] which may raise the inflammatory level of the brain parenchyma and activate the vascular cells. It seems that inflammatory stimuli in the peripheral blood and the brain parenchyma adjunctly activate the vascular cells. Activated vascular cells, in turn, could be a potential source of diffusible proinflammatory molecules such as prostaglandin E_2[18] and nitric oxide.[19] Thus, even low-grade inflammation associated with the pre-existing degenerative brain lesions may not only raise the basal inflammatory level of brain parenchyma but also enhance inflammatory signaling to brain parenchyma from the periphery.

Advanced age and the presence of brain disorders are considered to be the major risks for delirium, an acute confusional state that is triggered by a variety of systemic conditions.[10,11,20] The different extracerebral diseases that cause delirium include infection, trauma and major surgery, many of which are associated with systemic inflammation. We consider that the adjunctive pro-inflammatory effect of peripheral inflammation and parenchymal lesions to brain vasculature are a

part of the mechanism that increases the risk of delirium in patients with brain disorders. While strong systemic inflammation can induce central nervous system symptoms by itself,[8,9] mild to moderate systemic inflammation may also cause similar symptoms if the patients have pre-existing parenchymal lesions. Our results may therefore explain the vulnerability of neurological patients to delirium caused by inflammatory systemic conditions. Appropriate anti-inflammatory agents might, therefore, be a potential treatment of delirium in elderly neurological patients.

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Single Dose of OCH Improves Mucosal T Helper Type 1/T Helper Type 2 Cytokine Balance and Prevents Experimental Colitis in the Presence of Vα14 Natural Killer T Cells in Mice

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Background and Aims: Vα14 natural killer T (NKT) cells seem to play important roles in the development of various autoimmune diseases. However, the pathophysiologic role of NKT cells in inflammatory bowel disease remains unclear. To clarify the mechanism by which the activation of NKT cells mediates protection against intestinal inflammation, we investigated the antiinflammatory role of specifically activated Vα14 NKT cells by glycolipids in a mouse experimental model of colitis induced by dextran sulfate sodium (DSS).

Methods: Colitis was induced in C57BL/6 mice by the oral administration of 1.5% DSS for 9 days. A single dose of OCH or α-galactosylceramide, a ligand for NKT cells, was administered on day 3 after the induction of colitis. Body weights and colonic mucosal injury were assessed in each glycolipid-treated group. Interferon-γ and interleukin-4 levels in the supernatants from colonic lamina propria lymphocytes (LPLs) were measured by enzyme-linked immunosorbent assay.

Results: The administration of a single dose of OCH attenuated colonic inflammation, as defined by body weights and histologic injury. The protective effects of OCH could not be observed in Vα14 NKT cell-deficient mice. In vivo treatment with OCH had improved the interferon-γ/interleukin-4 ratio from colonic LPLs on day 9 after DSS treatment.

Conclusion: The present data indicated that the activation of Vα14 NKT cells by OCH plays a pivotal role in mediating intestinal inflammation via altered mucosal T-helper type 1/type 2 responses. Therapeutic strategies that are designed to activate specifically Vα14 NKT cells may prove to be beneficial in treating intestinal inflammation.

Key Words: colitis, natural killer T cells, OCH, T helper type 1/T helper type 2

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Natural killer T (NKT) cells have been identified as a novel lymphoid lineage distinct from conventional T cells and natural killer (NK) cells. NKT cells express both invariant Vα14 NKT-specific antigen receptor as well as an NK marker (NK1.1).[1–5] The specific features of this cell type include a limited repertoire with an invariant Vα chain consisting of the Vα14-Jα281 gene segment and the highly skewed Vβ chains Vβ8.2, Vβ7, and Vβ2 in mice. NKT cells are restricted by the nonclassical major histocompatibility complex class I-like molecule CD1d, which is expressed on cells of hematopoietic origin as well as on intestinal epithelial cells.[6–10] These cells recognize glycolipid antigens such as α-galactosylceramide (αGalCer), a glycolipid that is isolated from marine sponges that specifically binds CD1d.[11–14] NKT cells are abundant in the thymus, liver, and bone marrow, and are also found in peripheral lymphoid organs. It has been reported that NKT cells play an important role in various aspects of the immune response, including the regulation of allergic and autoimmune diseases[15–18] and the prevention of tumor metastasis.[19–22]

One of the mechanisms by which NKT cells elicit the effector function is through the production of large amounts of interferon (IFN)-γ, interleukin (IL)-4, and IL-10 in response to various stimuli.[1] In vitro and in vivo studies have shown that the cytokine profiles of these cells depend both on the nature of the activating stimulus and on the nature of the cytokines, and on other soluble factors in the local microenvironment. The activation of $NK1.1^+$T cells by CD3 cross-linking or CD1 results in the production of both IFN-γ and IL-4, whereas the stimulation of $NK1.1^+$ results in the production of IFN-γ

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only.[13,23,24] IL-12 stimulates NK1.1$^+$T cells to produce IFN-γ and inhibits their production of IL-4,[23,25,26] whereas IL-4 production by these cells requires IL-7 and is promoted by glucocorticoids.[27–29] Several costimulatory molecules play a role in the regulation of these cytokines. In the presence of blocking B7.2 (CD86) monoclonal antibody, αGalCer stimulation shifts the cytokine profile of NKT cells toward T helper (T_H) type 2 cells, whereas the presentation of αGalCer by CD40-activated antigen presenting cells causes a T_H1 shift of NKT cells.[30] Recently, Miyamoto et al[31] demonstrated that OCH, which has shorter hydrophobic chains than αGalCer, induces the production of, predominantly, IL-4 by NKT cells from murine spleen, leading to T_H2 bias in a Vα14 NKT cell-dependent manner. Therefore, the specific stimulation of NKT cells may have a therapeutic effect on various diseases associated with the T_H1-type or T_H2-type immune response.

The role of NKT cells in intestinal inflammation has been elucidated by several investigators. The ligand-specific activation of Vα14 NKT cells by αGalCer has been shown to protect mice against experimental colitis.[32] This protection was absent in CD1d$^{-/-}$ mice, and the elimination of NK1.1$^+$ cells reduced the effect of αGalCer. Other authors have reported that oxazolone-induced colitis, a T_H2-type colitis, is mediated by IL-13–producing NKT cells.[33] These results suggest that a CD1d–NKT cell interaction may be involved in the pathogenesis of colonic inflammation. However, the precise mechanism by which activated NKT cells modulate the pathogenesis of colitis is not yet understood. In the present study, we examined the role of NKT cells activated by OCH or αGalCer in protection against dextran sulfate sodium (DSS)-induced colitis. Our results indicate that the activation of Vα14 NKT cells by OCH shifted toward T_H2-type immune balance in the intestinal mucosa and that this is critical for protection against DSS-induced colitis.

MATERIALS AND METHODS

Mice

Specific pathogen-free C57BL/6 (B6) mice were purchased from Japan Clea (Tokyo, Japan). Jα281-deficient (Vα14 NKT cell-deficient [KO]) mice on a B6 background were generated, as described previously.[20] All mice were housed under specific pathogen-free conditions in microisolator cages in the animal facility at Hiroshima University, and only male mice (9 to 11 wk of age) were used.

DSS Colitis Model

DSS ([molecular weight, 5000] Wako Chemical Co, Osaka, Japan) was added to the water supply of the animals at a concentration of 1.5% (wt/vol) for days 1–9. The progression of colitis was monitored by a daily examination for rectal bleeding, perianal soiling, lack of grooming, hunched posture, weight loss, and mortality. Total body weight (in grams) was measured at the same time each day. All experiments were repeated at least twice with 5 to 15 mice.

In Vivo Injection of Glycolipid

OCH and αGalCer were first dissolved in dimethylsulfoxide at 100 μg/mL and then were diluted in phosphate-buffered saline (PBS) solution. To investigate the role of invariant NKT cells on the induction phase of DSS-induced colitis, each glycolipid (100 μg/kg in 200 μL of solution) was injected intraperitoneally on day 3 after the induction of colitis. Day 3 was selected because, in our preliminary studies, a single dose of glycolipids before the administration of DSS did not show any protective effect against DSS-induced colitis. Control animals received 200 μL of PBS solution containing the same concentration of dimethylsulfoxide (10%).

Assessment of the Severity of Colitis

Mice were killed on day 9 after DSS administration. Intestinal tissues were removed and opened longitudinally. The length of the colon was measured after the exclusion of the cecum and prior to division for histology. The tissues then were rolled concentrically and embedded in paraffin. Sections were stained with hematoxylin-eosin. The degree of inflammation of the colon was graded for severity according to mucosal damage (D) and the extension of the lesion (E) based on the method of Kitajima et al.[34] The histologic index was calculated as D plus E and was expressed as the mean of the score for each segment (i.e., for the cecum and the proximal, middle, and distal colon). The total score was the sum of the scores obtained in these sections. All slides were scored blindly.

Isolation of Colonic Lamina Propria Lymphocytes

Colonic lamina propria lymphocytes (LPLs) were isolated as described previously.[35] Briefly, nonadherent mesenteric tissues were removed, and the entire length of the intestine was opened longitudinally, washed with PBS solution, and cut into small (~5-mm) pieces. The dissected mucosa was incubated with $Ca^{++}Mg^{++}$-free Hanks balanced salt solution containing 1 mM ethylenediaminetetraacetic acid (Sigma, St. Louis, MO) for 20 minutes. Specimens were washed with Hanks balanced salt solution and then were incubated in 150 U/mL collagenase (Wako Chemical Co) in RPMI 1640 medium for 1.5 hours at 37 °C with stirring. Cells were suspended in 44% isotonic Percoll (Sigma) underlaid with 66% isotonic Percoll and were centrifuged for 20 minutes at 2200 revolutions per minute at room temperature. Cells at the interface were collected and washed twice with cold PBS. Approximately 2×10^6 cells per colon were recovered with >95% viability, as determined by trypan blue exclusion. Cells not excluding trypan blue were not included in the final count.

Cytokine Analysis in the Colonic Mucosa

Colonic LPLs were purified, transferred to 96-well plates (5×10^5 cells per well), and cultured for 48 hours in medium containing 500 ng/mL phorbol myristate acetate (PMA) (Sigma) and 50 ng/mL ionomycin (Sigma). After 48 hours, supernatants were harvested and stored at –20 °C until further analysis. The colon organ culture analysis for cytokines was performed as described previously.[36] Briefly, the mice were killed, the colon was removed, cut open longitudinally, and washed in PBS solution. The colonic tissue was transferred to 24-well flat-bottom culture plates containing fresh RPMI 1640 medium supplemented with penicillin and streptomycin, and was incubated at 37 °C for 24 hours. Culture supernatants were harvested and assayed for cytokines. IFN-γ, IL-4, and IL-10 were measured with OptEIA kits (BD, San Jose, CA). All samples were analyzed in triplicate.

Statistical Analysis

Data were analyzed with the Japanese version of StatView software (Hulinks, Tokyo, Japan) on a Macintosh Computer (Apple Computer, Cupertino, CA). The data are expressed as the mean ± SD. Differences between groups were examined for statistical significance with the Student *t* test after analysis of variance. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Efficacy of In Vivo Glycolipid Treatment in DSS-Induced Colitis

As reported previously, OCH, a relatively new synthetic analogue of αGalCer, induces the production of IL-4 by NKT cells from murine spleen, leading to T_H2 bias in a Vα14 NKT cell-dependent manner.[31] To investigate whether the specific activation of Vα14 NKT cells by OCH protects against colitis, a single dose of OCH was administered to C57BL/6 mice by intraperitoneal injection on day 3 during the induction of colitis. As shown in Figure 1A, OCH-treated mice lost significantly less weight compared with PBS-treated mice. Gross rectal bleeding was seen in 60% of PBS-treated mice (6/10 mice) and in 10% of OCH-treated mice (1/10 mice) on day 9. OCH significantly prevented shortening of the colon (Fig. 1B). Histologic analysis confirmed the presence of marked inflammatory cell infiltrations with a loss of the mucosal surface in the colons of mice injected with PBS (Figs. 2A, D). In contrast, mononuclear cell infiltration was observed, but colonic crypts were still conserved in the colons of OCH-treated mice (Figs. 2B, E). The histologic scores of the severity of colitis were significantly reduced in the OCH-treated group (Figs. 3A, B). PBS-treated and OCH-treated mice began to lose their initial body weight on day 6 and day 8, respectively (Fig. 1A), and all OCH-treated animals had histologic colitis on day 9. OCH may therefore delay colitis by 2 days rather than provide complete protection from colitis.

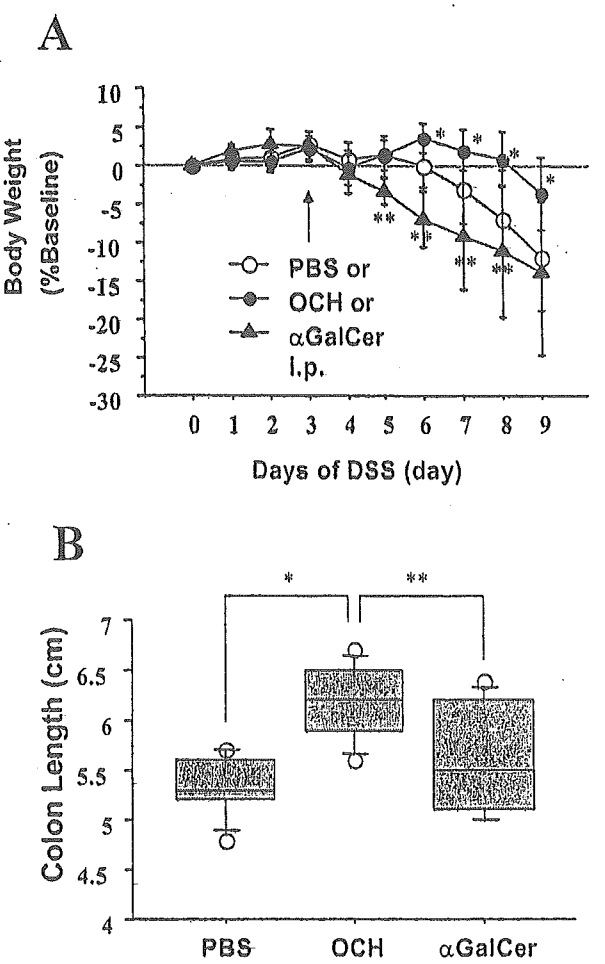

FIGURE 1. The effect of OCH on the protective immunity against DSS-induced colitis. A, C57BL/6 mice (n = 10 per group) were exposed continuously to 1.5% DSS in drinking water from day 0 to day 9. Mice were injected intraperitoneally with 100 μg/kg OCH, αGalCer, or PBS-solution on day 3. Body weights of individual mice were recorded daily. The measurement of body weight, as a percentage of starting weight, is shown. * = $P < 0.005$ compared with mice treated with PBS; ** = $P < 0.05$ compared with mice treated with PBS. B, Comparison of colon lengths in DSS-treated mice on day 9. Each box plot represents 10 mice. * = $P < 0.0001$ for a comparison of OCH versus PBS; ** = $P < 0.05$ for a comparison of OCH versus αGalCer.

A single dose of αGalCer also improved the histologic score in the middle and proximal parts of the colon at the same levels as OCH treatment (Figs. 2 and 3A). When the scores were pooled with differences in the other sites, OCH was superior to αGalCer in histology (Fig. 3B). Gross rectal bleeding was observed in 50% of αGalCer-treated mice (5/10 mice) on day 9. In total, αGalCer treatment resulted in no differences in body weight changes, colon length, or total histologic scores in comparison with PBS treatment (Figs. 1–3). These data demonstrated that OCH is more effective in preventing DSS-

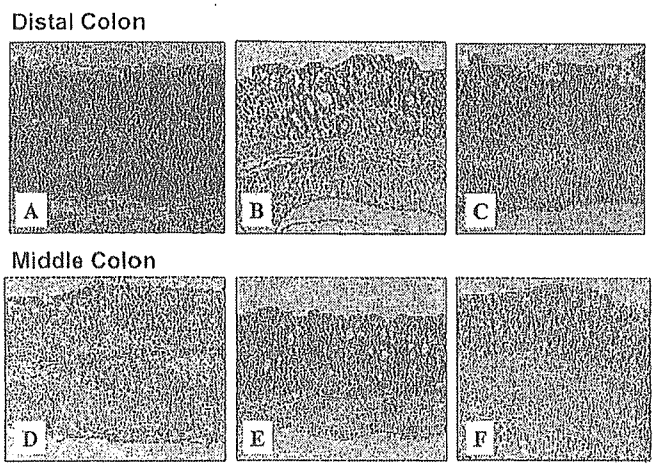

FIGURE 2. Histopathology of the colons from DSS-treated C57BL/6 mice. Representative photomicrographs (200×) of paraffin-embedded, hematoxylin-eosin–stained longitudinal sections of the distal parts of the colon (A–C) and the middle parts of the colon (D–F) from mice injected intraperitoneally with PBS solution (A and D), OCH (B and E), and αGalCer (C and F).

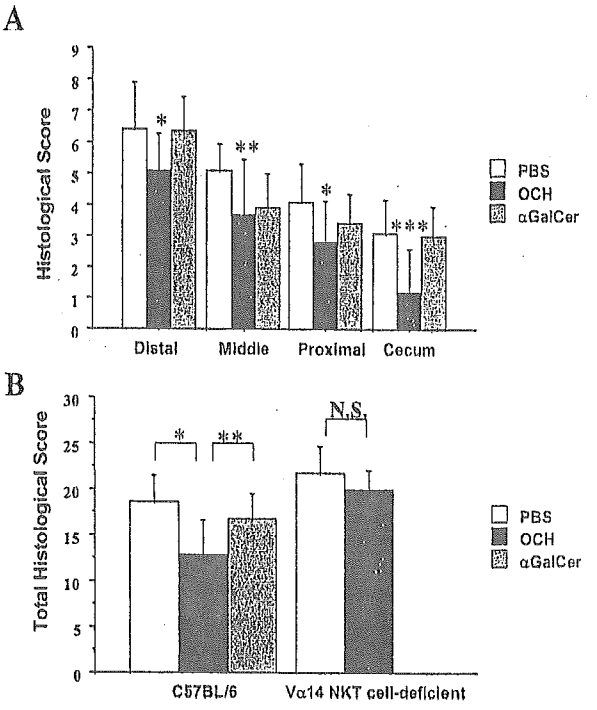

FIGURE 3. Histologic scores. Scoring was performed as described in "Materials and Methods." A, Histologic scores of each part of the colons. * = $P < 0.05$ for a comparison of PBS versus OCH; ** = $P < 0.05$ for a comparison of PBS versus OCH or αGalCer; and *** = $P < 0.005$ for a comparison of PBS versus OCH. B, Total histologic scores were expressed as the sum of the scores obtained in these sections. Vα14 NKT cell-deficient mice (n = 10 per group) also were treated with 1.5% DSS in drinking water from day 0 to day 9. The mice were injected intraperitoneally with 100 μg/kg OCH or PBS solution on day 3. * = $P < 0.005$ for a comparison of PBS versus OCH; ** = $P < 0.05$ for a comparison of OCH versus αGalCer in C57BL/6 mice; N.S. = not significant.

induced colitis than αGalCer. To confirm whether this protective effect of OCH was Vα14 NKT cell-dependent, we administered OCH to Vα14 NKT cell-deficient mice on day 3 during the induction of colitis. As shown in Figure 3B, OCH treatment had no effect on prevention of the development of DSS-induced intestinal inflammation in Vα14 NKT cell-deficient mice, as determined by evaluation of the total histologic score. These data indicated that the activation of Vα14 NKT cells by specific glycolipids influences protective immunity against intestinal inflammation.

Effect of Glycolipids on Mucosal Cytokine Balance

To examine whether the specific activation of Vα14 NKT cells by in vivo glycolipids could regulate the mucosal T_H1/T_H2 balance, we measured IFN-γ and IL-4 levels in supernatants from in vitro-stimulated colonic LPLs by enzyme-linked immunosorbent assay (ELISA) after the intraperitoneal injection of glycolipids. Colonic LPLs from DSS-treated C57BL/6 mice on days 5 and 9 produced significantly higher levels of both IFN-γ and IL-4 in comparison with those from non–DSS-treated, control, day 0 animals (Fig. 4A). The IFN-γ/IL-4 ratio increased in a time-dependent manner, suggesting that the progression of intestinal inflammation may be associated with T_H1-predominant immune responses (Fig. 4B). We next studied whether this ratio was affected by the administration of glycolipids. Treatment with both glycolipids induced higher amounts of IFN-γ and IL-4 than did PBS treatment (Fig. 4C, upper panels). When the IFN-γ/IL-4 ratio was calculated in each supernatant, both glycolipids significantly improved the ratio, and the degree of improvement by OCH was greater than that by αGalCer (Fig. 4C, lower panel). IL-10 had an anti-inflammatory effect on DSS-induced colitis.[37] We then analyzed IL-10 levels in the supernatants of colon organ cultures at an early phase after the injection of the glycolipids. Interestingly, OCH injection induced significantly higher IL-10 production than did αGalCer in the local colonic mucosa at 6 and 12 hours after injection (Figs. 5A, B). This IL-10 production was abrogated in Vα14 NKT cell-deficient mice, suggesting that the colonic mucosal IL-10 production is Vα14 NKT cell-dependent (data not shown). These data indicate that OCH induces a sufficient production of IL-10 in the local colonic mucosa and improves the subsequent mucosal T_H1/T_H2 cytokine balance at the time of development of colitis.

DISCUSSION

Here, we have shown that the specific activation of Vα14 NKT cells by OCH protects against DSS-induced colitis

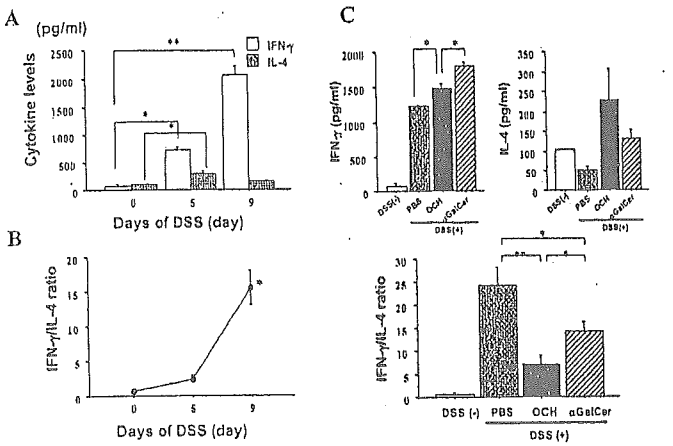

FIGURE 4. Comparison of the effects of glycolipids on mucosal cytokine balance in in vitro-stimulated colonic LPLs. A, C57BL/6 mice (n = 5 per group) were killed on the indicated days after 1.5% DSS administration, and colonic LPLs were purified. LPLs were stimulated in vitro with 500 ng/mL PMA and 50 ng/mL ionomycin, and were incubated at 37 °C for 48 hours. Supernatants were harvested, and cytokine levels were assessed by enzyme-linked immunosorbent assay. * = $P < 0.05$ for a comparison of day 0 versus day 5; ** = $P < 0.005$ for a comparison of day 0 versus day 9. B, Levels of IFN-γ and IL-4 were determined, and the IFN-γ/IL-4 ratio was calculated. * = $P < 0.005$ versus day 0. C, C57BL/6 mice treated with 1.5% DSS were injected with 100 μg/kg OCH, αGalCer, or PBS solution on day 3. The mice were killed on day 9, and colonic LPLs were purified. The LPLs were stimulated in vitro with 500 ng/mL PMA and 50 ng/mL ionomycin, and were incubated at 37 °C for 48 hours. Supernatants were harvested, and cytokine levels were analyzed by enzyme-linked immunosorbent assay. The amounts of IFN-γ and IL-4 were determined, and the IFN-γ/IL-4 ratio was calculated. DSS (−) and DSS (+) represent non-DSS-treated and DSS-treated mice, respectively. * = $P < 0.05$ for a comparison of OCH versus PBS or αGalCer, or PBS versus αGalCer. ** = $P < 0.01$ for a comparison of OCH versus PBS. Bars indicate the mean ± SD of 5 mice per group. The data are representative of 3 independent experiments.

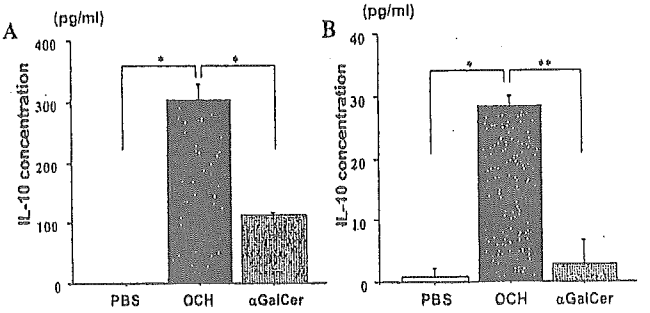

FIGURE 5. Rapid IL-10 induction after in vivo glycolipid injection during DSS administration. C57BL/6 mice treated with 1.5% DSS were injected with 100 μg/kg OCH, αGalCer, or PBS solution on day 3. The mice were killed 6 hours after injection (A) and 12 hours after injection (B), the colons were harvested, and organ culture was performed for cytokine analysis. The data are representative of 2 independent experiments. Bars indicate the mean ± SD of 5 mice per group. * = $P < 0.005$ for a comparison of OCH versus PBS or αGalCer; ** = $P < 0.05$ for a comparison of OCH versus αGalCer.

through the modulation of the mucosal T_H1/T_H2 cytokine balance.

It was recently reported that an analog of αGalCer, OCH, which has a truncated sphingosine chain, stimulates NKT cells to produce IL-4.[31] Therefore, OCH has the potential to elicit protective immunity against T_H1-mediated inflammatory disease. We have shown that a single dose of OCH attenuates DSS-induced colitis. This protection was mediated by Vα14 NKT cells because OCH did not elicit any protective effect in Vα14 NKT cell-deficient mice. In our preliminary study, we compared Vα14 NKT cell-deficient mice to wild-type B6 mice by administering 1.5% DSS. Interestingly, the knockout mice showed more severe intestinal inflammation when treated with DSS (our unpublished data). The loss of the protective effect of OCH in Vα14 NKT cell-deficient mice, however, may not be due to the increased susceptibility to DSS colitis because the effect of OCH was not observed when the knockout mice were given a lower dose (1.0%) of DSS (data not shown). These findings suggest that the specific activation of Vα14 NKT cells could reduce intestinal inflammation.

In contrast, a single dose of αGalCer, which was originally discovered as a ligand for NKT cells, had a smaller effect on prevention against DSS-induced colitis than OCH. Why do these glycolipids differ in the ability to protect against intestinal inflammation?

To examine the effects of these glycolipids on local immunologic responses, the levels of cytokines produced by colonic LPLs were analyzed. Colonic LPLs from DSS-treated C57BL/6 mice produced a significantly higher IFN-γ/IL-4 ratio in comparison to that from non–DSS-treated control animals. This ratio increased in a time-dependent fashion. We found in the present study that this ratio was significantly decreased by treatment with glycolipids, and OCH improved the ratio more significantly than αGalCer. The severity of the disease inversely correlated with the IFN-γ/IL-4 ratio. Therefore, OCH may prevent colitis through improvement of the mucosal T_H1/T_H2 cytokine balance.

The T_H1/T_H2 response in DSS-induced colitis remains unclear. It has been reported that DSS-induced colitis in C57BL/6 mice is characterized by a T_H1-type response with a strong induction of IFN-γ messenger RNA expression.[38] In DSS-treated mice, anti–IFN-γ and/or anti-tumor necrosis factor-α antibodies significantly reduce the severity of colitis.[39] On the other hand, the role of IL-4 in DSS-induced colitis is not fully understood. Stevceva et al[40] showed that DSS-induced colitis is ameliorated in IL-4–deficient mice, suggesting that even IL-4 may play a pathologic role in the intestinal inflammation induced by DSS. Therefore, the beneficial effects of the

glycolipids in the DSS-induced colitis model may not be due simply to an increase in IL-4 production.

Another T_H2-related cytokine, IL-10, has been widely characterized as an immunosuppressive cytokine and is important for mucosal immunologic homeostasis.[41–43] We also detected that OCH rapidly induces the localized expression of IL-10 in the colonic mucosa. How does OCH induce secretion of IL-10? Although we could not determine which cells in the colonic mucosa produce IL-10 in response to the activation of NKT cells by OCH, dendritic cells are one of the main producers of IL-10,[44] and CD1d-restricted NKT cells are known to contribute to immune function by regulating dendritic cell maturation.[45] A recent study showed that αGalCer stimulates the production of IL-12 by dendritic cells.[46] OCH has shorter hydrophobic chains than does αGalCer but has the same hydrophilic cap.[31] Therefore, OCH may bind to the CD1d groove less stably and may induce a weaker T-cell receptor signal to CD1d-restricted T cells than does αGalCer. Such T cells could induce antiinflammatory mature dendritic cells that produce more IL-10 than IL-12.[47] We hypothesized that the difference in the affinities of the glycolipids for the CD1d groove may influence the development of mature dendritic cells that preferentially produce IL-12 or IL-10. Since the murine colonic mucosa contains dendritic cells, which have a capacity to produce both IL-10 and IL-12,[48] it would be important to know whether these dendritic cell-derived cytokine balances are affected by activating NKT cells with glycolipids. Further studies are needed to clarify the precise mechanism underlying the protective immunity induced when NKT cells are activated.

It was recently shown that a course of multiple injections of αGalCer provides some protection against DSS-induced colitis.[32] Our present findings indicated that a single injection of αGalCer is not sufficient to elicit a preventive effect against DSS-induced colitis. This difference may be explained by the fact that single and multiple injections of αGalCer are known to induce specifically T_H1-predominant[49,50] and T_H2-predominant[51–53] immune responses, respectively. Miyamoto et al[31] reported that a single injection of OCH, but not of αGalCer, improves experimental allergic encephalomyelitis, a T_H1-associated disease, in mice. These previous findings and the results of the present study suggest that shifting toward a T_H2-type mucosal immune response may be crucial for protecting against DSS-induced colitis.

Heller et al[33] showed that oxazolone-induced colitis, a T_H2 colitis model, is mediated by IL-13–producing NKT cells. Whether NKT cells act as effector cells or regulatory cells may depend on the pathophysiology of the disease. Recently, Fuss et al[54] demonstrated the presence of IL-13–producing nonclassical NKT cells in the colonic mucosa of patients with ulcerative colitis. It would be interesting to examine the interaction between these pathogenic noninvariant mucosal NKT cells and anti-inflammatory invariant NKT cells. According to our present study, OCH may have potential as a treatment of human T_H1-predominant intestinal inflammatory diseases, such as Crohn's disease.[55]

In summary, we showed that Vα14 NKT cells are important for attenuating DSS-induced colitis. This protective immunity may be modulated by the activation status of Vα14 NKT cells. Although additional experiments are needed, our data indicate that an early and sufficient T_H2-biased immune response in the intestinal mucosa during the onset of colitis may have an antiinflammatory effect. Future studies with Vα14 NKT cell-deficient mice and analyses of the effects of glycolipids in other animal models of colitis will clarify our understanding of the pathologic process underlying colitis and will improve the chances of developing effective treatments for human inflammatory bowel disease.

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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]

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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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[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.

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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

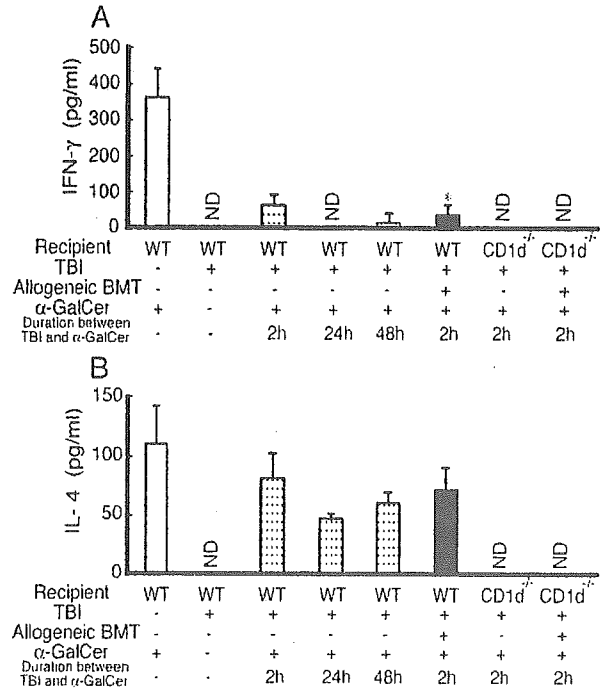

FIGURE 1. Cytokine responses to α-GalCer in lethally irradiated mice with or without BMT. WT and CD1d$^{-/-}$ B6 mice received 13 Gy TBI. Two, 24, or 48 h later, mice were injected i.p. with α-GalCer (100 μg/kg) or diluent. A cohort of animals were transplanted with allogeneic BM cells (5×10^6) and spleen 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.

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*).

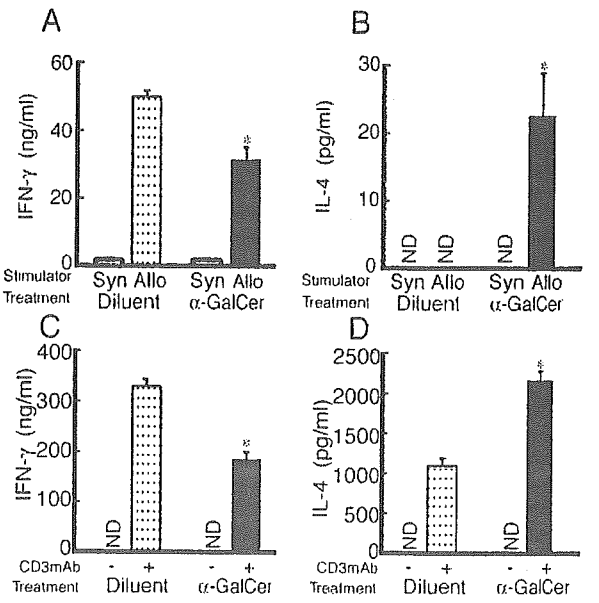

FIGURE 2. Administration of α-GalCer to recipients of allogeneic BMT polarizes donor T cells toward Th2 cytokine secretion. Lethally irradiated (13 Gy) B6 mice were transplanted with BM cells (5×10^6) and spleen cells (5×10^6) isolated from BALB/c mice, followed by injection of either α-GalCer or control 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.

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

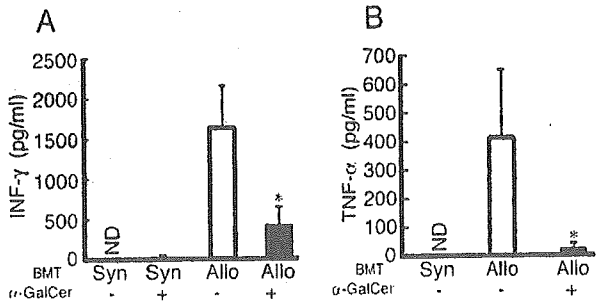

FIGURE 3. A single injection of α-GalCer to recipients of allogeneic BMT markedly reduces serum levels of IFN-γ and TNF-α. WT B6 mice were transplanted as in Fig. 2. Sera (n = 3–10/group) were obtained from diluent-treated (□) and α-GalCer-treated (■) recipients on day 6 after BMT, and serum levels of 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

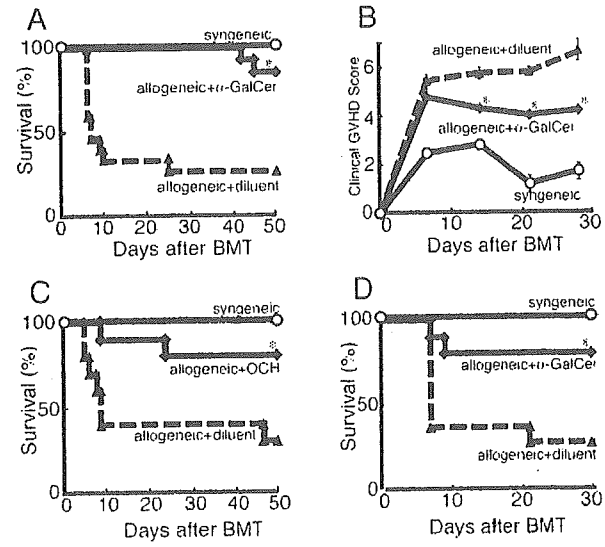

FIGURE 4. A single injection of NKT ligands to BMT recipients modulates acute GVHD. BMT was performed as in Fig. 2. *A*, Survival curves of syngeneic control group (○, solid line; n = 9); allogeneic, diluent-treated recipients (▲, dotted line; n = 15); and allogeneic, α-GalCer-treated recipients (◆, 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.

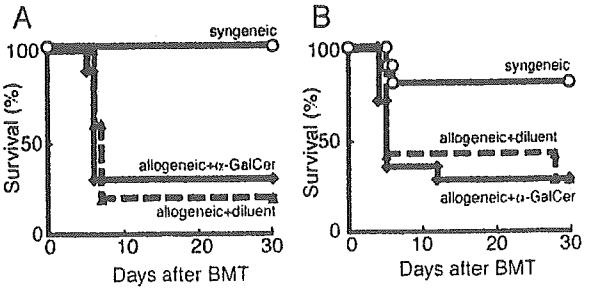

FIGURE 5. Host NKT cells and host IL-4 production are required for suppression of GVHD by α-GalCer. *A*, Lethally irradiated CD1d$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line;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

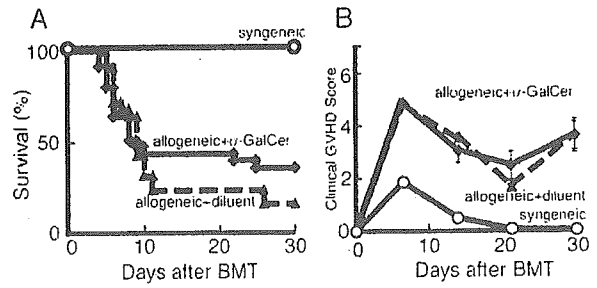

FIGURE 6. The protective effects of α-GalCer against GVHD are dependent upon the STAT6 pathway of donor T cells. Lethally irradiated B6 mice were transplanted with TCD-BM cells (4 × 10^6) from WT BALB/c mice and spleen cells (5 × 10^6) from STAT6$^{-/-}$ BALB/c mice. *A*, Survival curves of the syngeneic 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.

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