β-Glycosphingolipids-mediated lipid raft alteration is associated with redistribution of NKT cells and increased intrahepatic CD8$^+$ T lymphocyte trapping

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Abstract **The aim of this study was to determine the effect of β-glycosphingolipids on intra-hepatic natural killer T (NKT) lymphocyte regulatory function and on lymphocyte trapping via alteration of cell membrane lipid rafts. Immune-mediated colitis was induced by intracolonic instillation of trinitrobenzene sulfonic acid. Mice were treated with β-lactosylceramide (LC), β-glucosylceramide (GC), β-galactosylceramide, ceramide, or a combination of both GC and LC (IGL), or solvent alone. Lipid rafts were investigated by fluorescence-activated cell sorting analysis of ganglioside-GM1 and fluorescence microscopy of structure. Administration of β-glycosphingolipids resulted in an increased intrahepatic/peripheral NKT ratio, increased intrahepatic CD8+ lymphocyte trapping, decreased serum interferon-γ (IFN-γ) levels and decreased serum IFN-γ/interleukin-10 ratio. Administration of GC, LC, or IGL significantly altered the levels of GM1, a key marker of lipid rafts, on NKT regulatory lymphocytes. The immune modulatory effect of β-glycosphingolipids was associated with increased survival and significant alleviation of colitis as determined by improvement in both the macroscopic and microscopic scores. In conclusion, administration of β-glycosphingolipids increased NKT regulatory lymphocyte redistribution and intrahepatic CD8$^+$ T lymphocyte trapping, resulting in alleviation of immune-mediated colitis. The effects of these naturally occurring compounds were associated with modification of the T lymphocyte lipid raft structure, which is a site for immune modulation.**—Lalazar, G., A. Ben Ya'acov, N. Eliakim-Raz, D. M. Livovsky, O. Pappo, S. Preston, L. Zolotarov, and Y. Ilan. **β-Glycosphingolipids-mediated lipid raft alteration is associated with redistribution of NKT cells and increased intrahepatic CD8$^+$ T lymphocyte trapping.** ***J. Lipid Res.*** **2008.** 49: **1884–1893.**

Supplementary key words liver tolerance • lipid rafts • natural killer T cell

The unique lymphocyte environment provided by the liver favors tolerogenic immune responses (1). During systemic immune responses, activated CD8$^+$ T lymphocytes selectively accumulate and undergo apoptosis in the liver (2). Activated T cell trapping is believed to play a part in liver-mediated tolerance induction (3).

Natural killer T (NKT) cells, a subset of regulatory lymphocytes, are considered players in the innate immune system (4). Via their T cell receptor (TCR), these cells recognize glycosphingolipids anchored by a ceramide tail to CD1d, which is a major histocompatibility complex (MHC), class I-like molecule (5). The liver is unique in that it harbors a higher percentage of NK and NKT cells than other organs (6). In the murine liver, as many as 40% of lymphocytes may be classical NKT cells. The role of these cells in diverse neoplastic, inflammatory, and infectious processes has been demonstrated (7). NKT function is important in several diseases, such as hepatocellular carcinoma, concanavalin A-induced hepatitis, and experimental colitis (8–10).

NKT lymphocytes are influenced by a variety of endogenous ligands and environmental stimuli, including disease target antigens, antigen-presenting cells (APCs), costimulatory signals, soluble factors, and effector cells (5). The functions of NKT lymphocytes are controlled by affinity thresholds for glycosphingolipid antigens that may have an important role in cell activation (11). Studies on immune recognition of carbohydrates and lipids are based on the identification of precise structures recognized by the im-

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Abbreviations: APC, antigen-presenting cell; CTx, cholera toxin; FACS, fluorescence-activated cell sorting; α-GalCer, α-galactosylceramide; GC, β-glucosylceramide; GLC, β-galactosylceramide; IFN-γ, interferon-γ; IGL, β-glucosylceramide + β-lactosylceramide; IL-4, interleukin-4; LC, β-lactosylceramide; MHC, major histocompatibility complex; NKT cell, natural killer T cell; TCR, T cell receptor; TNBS, trinitrobenzene sulfonic acid.

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mune system and on the production of the biologically relevant epitopes by synthetic chemistry. New immunotherapy approaches of interfering with glycosphingolipid metabolism or directly supplementing rationally designed glycosphingolipids are being explored (12).

The only efficient means to selectively stimulate NKT cells in vivo is by the sea sponge-derived α-galactosylceramide (α-GalCer). Multimers of CD1d1 α-GalCer and α-GalCer analog-loaded complexes demonstrate cooperative engagement of the iNKT cell Vα14Jα18 receptor (13). Administration of α-GalCer to mice results in potent activation of NKT cells, rapid and robust interleukin-4 (IL-4) and interferon-γ (IFN-γ) secretion, and activation of a variety of innate and adaptive immune cells (13). Several glycosphingolipids and phospholipids derived from mammalian, bacterial, protozoan, and plant species have been identified as potential natural NKT ligands. The semi-invariant αβTCRs recognize iGb3, a mammalian glycosphingolipid, as well as microbial α-glycuronylceramides found in the cell walls of Gram-negative, lipopolysaccharide-negative bacteria (14, 15). This glycosphingolipid was proposed to be recognized by NKT cells under pathophysiological conditions such as cancer and auto-immune disease. Recent data suggested that it may not be the natural ligand for these cells (16). The presentation of a neo-self glycosphingolipid by infectious assault of APCs activates iNKT cells, which, in turn, release pro-inflammatory or anti-inflammatory cytokines to jump-start the immune system (7, 17, 18).

β-Glycosphingolipids are naturally occurring intermediates of complex glycosphingolipids and are found within cell membranes (19). In vitro, CD1d-bound β-glucosylceramide (GC) inhibits NKT cell activation by α-GalCer (20). GC synthase deficiency leads to defective ligand presentation by CD1d, thus inhibiting NKT activation. β-D-GalCer-deficient mice exhibit normal NKT cell development and function, and cells from these animals stimulate NKT hybridomas (19). In striking contrast, the same hybridomas fail to react to CD1d1 expressed by a GC-deficient cell line. Administration of GC in vivo attenuated the NKT-mediated damage in concanavalin A-induced hepatitis, immune-mediated colitis, and animal models of diabetes (8, 21, 22).

Lipid rafts are 50 nm structures that contain specialized glycosphingolipids and cholesterol. They are enriched in glycosylphosphatidylinositol, and play an important role in intracellular trafficking (23). The role of lipid rafts in immune modulation involves concentration of specific membrane proteins and lipids in membrane microdomains (24). Small changes in partitioning of these rafts initiate a signaling cascade. In normal T cells, ligation of the TCR induces rapid lipid raft clustering that leads to concentration of signaling proteins at the area of contact between APCs and T cells (25). Murine CD1d (mCD1d) is localized at the lipid rafts, and disruption of lipid raft structures may block efficient signaling through this molecule (26). It was suggested that partitioning of mCD1d into membrane rafts increases the capacity of APCs to present limiting quantities of glycosphingolipid antigens, perhaps by stabilizing mCD1d/antigen structures on plasma membranes and optimizing TCR engagement on NKT cells (27).

The aim of this study was to determine the effect of naturally occurring β-glycosphingolipids on liver-mediated immune regulation in a murine model of experimental colitis. It was found that administration of β-glycosphingolipids altered NKT cell redistribution and intrahepatic $CD8^+$ T lymphocyte trapping. This effect was associated with modification of the T lymphocyte lipid raft structure. These immune modulatory effects were associated with a Th1-to-Th2 cytokine shift and significant alleviation of experimentally induced colitis.

MATERIALS AND METHODS

Preparation of glycosphingolipids

GC, β-galactosylceramide (GLC), and β-lactosylceramide (LC) were purchased from Avanti Polar Lipids (Alabaster, AL). GC is a natural, soybean-derived compound with a chemical purity of >99%. LC (D-lactosyl-b1-*N*-dodecanoyl-D-erytro-sphingosine, C12 β-D-lactosyl ceramide) is a synthetic compound. Ceramide was purchased from Alexis Biochemicals (San Diego, CA). Glycosphingolipids were dissolved in ethanol and emulsified in PBS.

Animals

Twelve week-old male C57Bl/6 mice were obtained from Harlan Laboratories (Jerusalem, Israel) and maintained in the Animal Core of the Hadassah-Hebrew University Medical School. Mice were administered standard laboratory chow and water ad libitum and were maintained in a 12 hour light/dark cycle. Animal experiments were carried out according to the guidelines of the Hebrew University-Hadassah Institutional Committee for Care and Use of Laboratory Animals. All experiments were approved by the committee.

Induction of experimental colitis

Colitis was induced by rectal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma) at a concentration of 1.5 mg/mouse. The TNBS was dissolved in 100 μl of 50% ethanol as described previously (28). Rectal instillations were performed on the first and the fifth days of the experiment.

Experimental groups

Twelve experimental and control groups containing twelve mice per group were studied (**Table 1**). Mice in experimental groups A–F were injected with TNBS. Mice in group A were administered intra-peritoneal injections of 100 μl PBS daily for 12 days.

TABLE 1. Experimental groups

Group	TNBS	Glycosphingolipid
A	+	PBS
B	+	β-glucosylceramide (GC)
C	+	β-lactosylceramide (LC)
D	+	β-galactosylceramide (GLC)
E	+	β-glucosylceramide + β-lactosylceramide (IGL)
F	+	Ceramide
G	−	PBS
H	−	β-glucosylceramide (GC)
I	−	β-lactosylceramide (LC)
J	−	β-galactosylceramide (GLC)
K	−	β-glucosylceramide + β-lactosylceramide (IGL)
L	−	Ceramide

TNBS, trinitrobenzene sulfonic acid.

Mice in groups B, C, D, E, and F were given daily intraperitoneal injections of GC, LC, GLC, ceramide, or IGL, which is a 1:1 combination of GC and LC, respectively (1 μg in 100 μl PBS). Naïve mice in groups G–L were similarly injected with the respective glycosphingolipids in the absence of TNBS administration. Animals were euthanized on day 10.

Isolation of splenocytes and intrahepatic lymphocytes

Splenocytes and intrahepatic lymphocytes were isolated as described (29). In brief, livers and spleens were placed in RPMI-1640 + 5% fetal calf serum (FCS). The spleens were mechanically disrupted using a 70 μm nylon cell strainer (Falcon), and the debris was removed by centrifugation (1,250 rpm for 7 min). Red blood cells were lysed with 1 ml of cold 155 mM ammonium chloride lysis buffer and immediately centrifuged (1,250 rpm for 3 min). Splenocytes were then washed and resuspended in 1 ml RPMI + 5% FCS. A viability of greater than 90% was determined by trypan blue staining. For intrahepatic lymphocyte isolation, livers were mechanically disrupted through a stainless steel mesh (size 60; Sigma), and the debris in the cell suspension was allowed to settle for 5 min. Ten milliliters of Lymphoprep (Ficoll; Axis-Shield PoC AS, Oslo, Norway) was slowly layered beneath an equal volume of cell suspension in 50 ml tubes. The samples were centrifuged at 1,800 rpm for 18 min. Cells at the interface were collected and centrifuged again at 1,800 rpm for 10 min to obtain a pellet of cells depleted of hepatocytes. The cell pellet was resuspended to a final volume of 250 μl. Approximately 1×10^6 cells/mouse liver were recovered.

Cytokine measurement

Serum IFN-γ, IL-12, IL-4, and IL-10 levels were measured by ELISA using commercial kits (Genzyme Diagnostics).

Isolation of lipid rafts

Flotation studies were performed as described (30). Aliquots of 3×10^7 freshly isolated splenocytes were washed three times in ice-cold PBS. Cells were lysed in TNE buffer (150 mM NaCl, 25 mM Tris-HCl, and 5 mM EDTA, pH 7.5) containing 1% Triton X-100 for 30 min at 4°C. Supernatants were transferred to TLS-55 centrifuge tubes (Beckman Instruments) and adjusted to 35% Nycodenz (Sigma) by adding an equal volume of ice-cold 70% Nycodenz dissolved in TNE buffer. A linear step gradient of 8–25% Nycodenz in TNE buffer was added above the lysate, and the samples were centrifuged at 55,000 rpm for 4 h at 4°C. Twelve 180 μl fractions were collected sequentially and stored at −20°C until immunoblot analysis.

Immunoblot

For detection of ganglioside GM1 using dot blot, 10 μl of each flotation fraction was supplemented with 90 μl PBS and blotted onto Nytran nylon membranes (Schleicher and Schuell) using a vacuum dot blotter (Bio-Rad). Dried membranes were blocked with 5% BSA in PBS and incubated with cholera toxin B (CTxB)-HRP (Sigma; 12.5 ng/ml) for 30 min, followed by four washes with Tris-buffered saline-Tween-20. Membranes were developed using Western blot luminol reagent (Santa Cruz Biotechnology).

Flow cytometry for GM1 and lymphocyte subsets

Following lymphocyte isolation, flow cytometry was performed on 1×10^6 lymphocytes in 100 μl PBS. GM1 detection was performed by staining the cells with CTxB-FITC. For determination of the percentage of NKT lymphocytes, PE-Cy5 anti-mouse CD3 and PE anti-mouse NK1.1 antibodies were used (eBioscience). $CD4^+$ and $CD8^+$ subsets were detected with PE-Cy5 anti-mouse CD3 and PE-anti-mouse CD4 or CD8. Cells were incubated for 30 min at 4°C in the dark. The cells were then washed and resuspended in 200 μl PBS. Analytical cell sorting was performed on 1×10^4 cells from each group using a fluorescence-activated cell sorter (FACSTAR plus; Becton Dickinson, Oxnard, CA). Only live cells were counted, and unstained cells served as a control for background fluorescence. Gates were set on forward- and side-scatters to exclude dead cells and red blood cells. Data were analyzed with the Consort 30 two-color contour plot program (Becton Dickinson) or the CELLQuest 25 program.

Characterization of isolated lipid microdomains

After incubation of 5×10^6 splenocytes with 20 μg/ml CTxB-FITC in 0.1% BSA in PBS at 4°C for 30 min in the dark, GM1 clustered on their surface. Cells were washed and fixed in 3.7% paraformaldehyde for 15 min at room temperature, then mounted on poly-L-lysine-coated microscope slides with vectashield (Vector Laboratories). For splenocyte labeling, 2.5×10^6 splenocytes were stained with 2 μg of CTxB-HRP in 0.1% BSA in 1 ml PBS for 30 min. Cells were lysed with 200 μl splenocyte lysis buffer (0.5% Triton X-100, 0.23% deoxycholate, 10 mM EDTA, 10 mM Tris, pH 7.5, 100 mM NaCl) for 15 min on ice. The lysates were centrifuged (2,000 rpm for 5 min at 4°C). A 20 μl aliquot containing approximately 2.5×10^6 cells was supplemented with 100 μl PBS and blotted onto a Nytran membrane using a vacuum dot blotter. GM1 was detected using the Western blot luminol reagent. An equal number of cells (5×10^6) were used for the GM1 clustering assay.

Grading of histological lesions

For histological evaluation of inflammation, distal colonic tissue (last 10 cm) was removed and fixed in 10% formaldehyde. Five paraffin sections from each mouse were stained with hematoxylin-eosin using standard techniques. The degree of inflammation was determined upon microscopic analysis of cross sections of the colon and was graded semi-quantitatively on a scale from 0 to 6 as described, based on signs of inflammation, marked infiltration with high vascular density and bowel wall thickening, and disruption of normal bowel architecture (8). Grading was performed by two experienced and blinded examiners. The extent of disease was assessed by evaluation of the entire bowel taken from each animal.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. A value of $P < 0.05$ was considered significant. ANOVA was used for comparison between the groups when applicable.

RESULTS

Effect of β-glycosphingolipids on splenic and intrahepatic NKT lymphocyte distribution

Administration of β-glycosphingolipids was associated with alteration of the intrahepatic NKT lymphocyte number. This effect was dependent on the immune microenvironment and was most profound in the mice with experimental colitis. Both GC and IGL were associated with a significant increase in the number of intrahepatic NKT lymphocytes (22% and 19% for groups B and E, respectively vs. 13% for group A; $P < 0.005$; **Fig. 1A**). In contrast, administration of LC, GLC, or ceramide was associated with a decrease

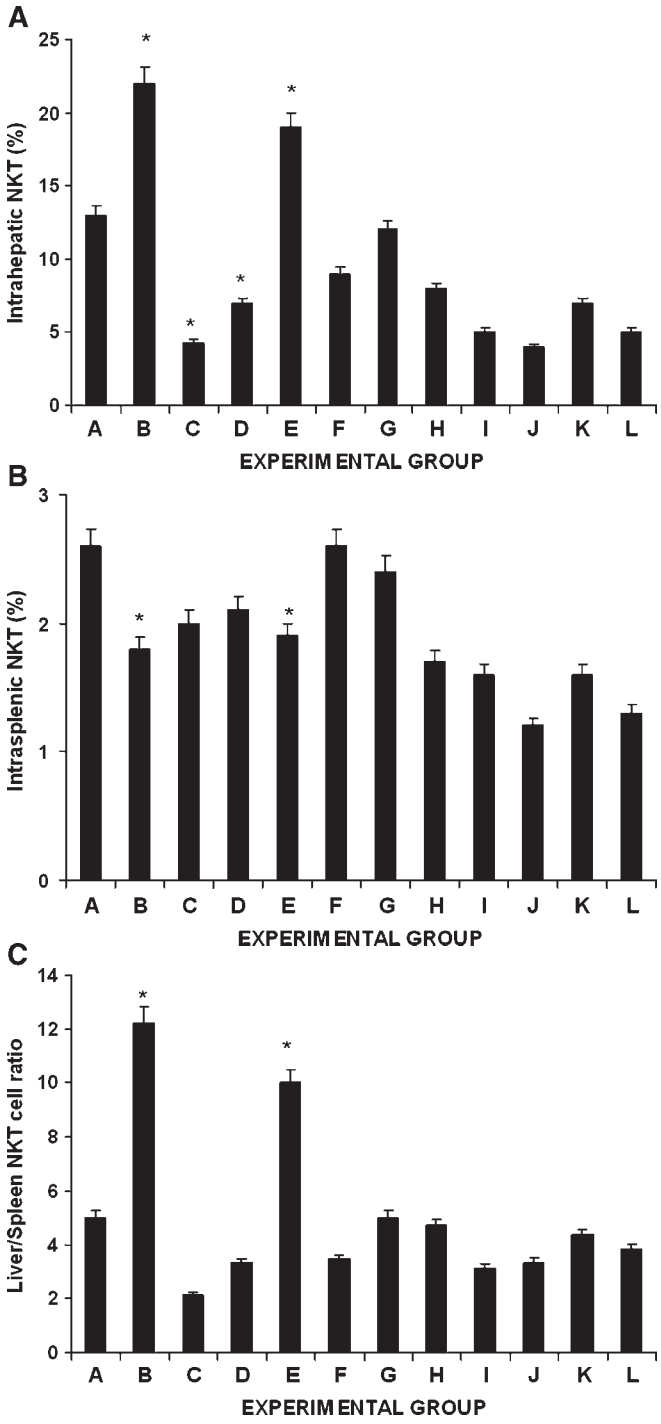

Fig. 1. Effects of β-glycosphingolipids on splenic and intrahepatic natural killer T (NKT) lymphocytes. A: Flow cytometry analysis of intrahepatic lymphocytes harvested from mice treated with β-glycosphingolipids. Both β-glucosylceramide (GC) and β-glucosylceramide + β-lactosylceramide (IGL) were associated with a significant increase in the number of intrahepatic NKT lymphocytes. In contrast, administration of β-lactosylceramide (LC), β-galactosylceramide, or ceramide was associated with a decrease in intrahepatic NKT cell number. B: Fluorescence-activated cell sorting (FACS) analysis was performed on isolated intrasplenic lymphocytes from mice that had been treated with β-glycosphingolipids. No significant effect on the peripheral NKT cell number is noted. C: A calculated ratio of the intrahepatic-to-intrasplenic NKT cells showing a significant increase for mice treated with GC and IGL. All mice in all groups were studied; error bars represent SD. * $P < 0.05$.

in intrahepatic NKT cell number (4%, 7%, and 9%, for groups C, D, and F, respectively). These results suggest that GC administration can overcome the suppressive effect of LC on NKT cells in this setting. No significant effect on the peripheral NKT cell number was noted by fluorescence-activated cell sorting (FACS) analysis of intrasplenic NKT lymphocytes (Fig. 1B). The calculated intrahepatic-to-intrasplenic NKT ratio was significantly increased for mice treated with GC and IGL (12.22 and 10.00 in groups B and E, vs. 5.00 for mice in group A, respectively; $P < 0.005$; Fig. 1C). β-Glycosphingolipid treatment did not lead to a significant change in the NKT ratio in naïve animals.

Effect of β-glycosphingolipids on intrasplenic and intrahepatic CD4-to-CD8 lymphocyte ratios

To determine the role of the liver in $CD8^+$ lymphocyte trapping, FACS analysis was performed on $CD4^+$ and $CD8^+$ T lymphocytes from all animals in treated and control groups. The number of intrahepatic $CD8^+$ cells decreased following colitis induction (11% for group A vs. 15% for group G; $P < 0.005$). Administration of GC and IGL was associated with a significant increase in intrahepatic $CD8^+$ cells (21% and 19% for groups B and E, respectively; $P < 0.005$ compared with group A). A lesser effect was noted on CD4 cells (4% and 2% for groups B and E, respectively). For each group, the CD4-to-CD8 lymphocyte ratios for the spleen and liver were calculated separately. A second ratio of the splenic-to-intrahepatic CD4/CD8 ratios was also determined (**Fig. 2**). Induction of experimental colitis was associated with a decrease in intrahepatic $CD8^+$ lymphocyte trapping (ratio of 0.55 vs. 0.61 for groups A and G, respectively; $P < 0.005$). Administration of GC and IGL was associated with a significant increase in this ratio (0.95 and 0.83 vs. 0.55 for group B, E, and A, respectively; $P < 0.005$; Fig. 2). This result suggests increased intrahepatic $CD8^+$ lymphocyte trapping. A smaller effect was noted in mice treated with GLC or ceramide.

Effect of β-glycosphingolipids on serum cytokine levels

Treatment with β-glycosphingolipids was associated with a profound alteration of the Th1 and Th2 immune balance in animals with colitis. Serum IFN-γ levels decreased significantly for animals treated with GC and IGL, compared with untreated controls (135 and 110 pg/ml vs. 455 pg/ml for groups B, E, and A, respectively; $P < 0.005$; **Fig. 3A**). Serum IL-10 levels decreased in GC- and IGL-treated mice (27 and 27.5 pg/ml for groups B and E, respectively, vs. 35 pg/ml for group A; $P < 0.005$). To assess the effect of GC and IGL on the Th1/Th2 immune paradigm, the IFN-γ/IL-10 ratio was calculated. Administration of GC and IGL led to a significant reduction in this ratio (5.0, 4.0 in groups B and E, respectively, vs 13.3 in group A ($P < 0.005$; Fig. 3B). No significant effect was noted for serum IL-4 and IL-12. These data suggested that the β- glycosphingolipid-altered NKT regulatory lymphoycte distribution and intrahepatic $CD8^+$ lymphocyte trapping were associated with a shift from a Th2 to a Th1 immune response in the TNBS colitis model.

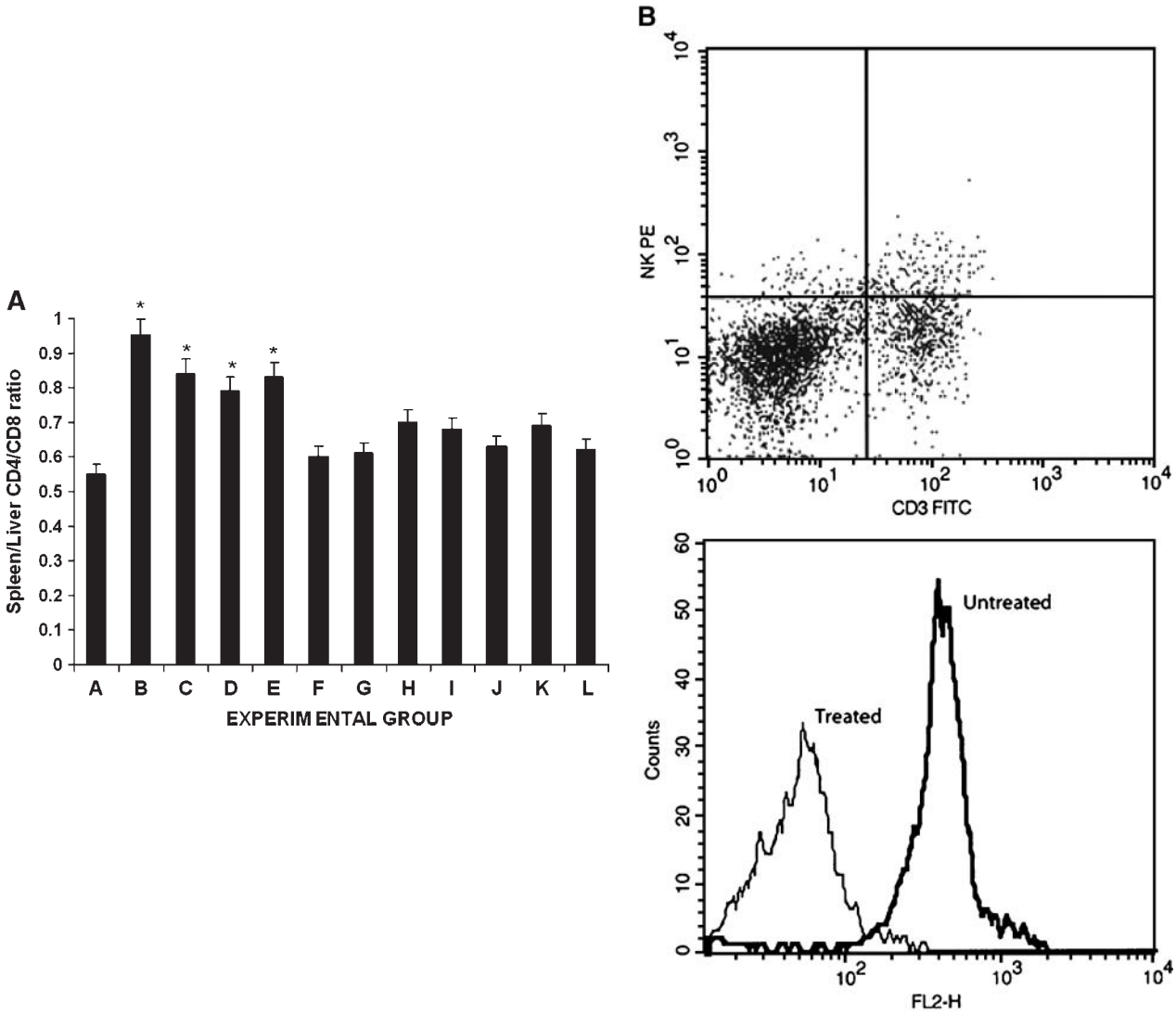

Fig. 2. Effects of β-glycosphingolipids on $CD4^+$ and $CD8^+$ lymphoycte distribution. A: The intrasplenic-to-intrahepatic $CD4^+$ to $CD8^+$ lymphocyte ratio was determined via FACS analysis on isolated intrahepatic and intrasplenic lymphocytes. A ratio between each of the splenic to intrahepatic CD4/CD8 ratios was calculated. Induction of experimental colitis was associated with a decrease in intrahepatic $CD8^+$ lymphocyte trapping, whereas the administration of GC and IGL was associated with a significant increase in this ratio. * $P < 0.05$. B: A representative FACS analysis plot of LC-treated mice (group C) versus control group A. The intrahepatic NKT cell population decreased in this group.

Effect of β-glycosphingolipids on lipid rafts and cell membranes

GM1 content in the detergent-insoluble fractions and in the detergent-soluble (cytosolic) fractions was analyzed by dot blot analysis (**Fig. 4**). In TNBS-treated mice, administration of GC, LC, and IGL resulted in alteration of both the lipid raft and cytosolic fractions. In contrast, administration of β-glycosphingolipids was not associated with a significant change in GM1 content in naïve mice.

To determine the effect of distinct β-glycosphingolipids on lipid rafts in NKT lymphocytes, FACS analysis of GMI expression on the cell surface was performed using CTx-FITC. Administration of TNBS led to a major decrease of GM1 expression in $CD4^+$, $CD8^+$, and NKT cells (24%, 26%, and 7.55%, vs. 19%, 20%, and 4.6%, for CD4+, CD8+, and NKT+ in naïve vs. TNBS, respectively) . Furthermore, administration of GC, LC, and IGL was associated with an additional decrease in GM1 expression on $CD8^+$ and $CD4^+$ lymphocytes but not on NKT cells (**Fig. 5**, $P < 0.005$). These effects were not observed in naïve animals.

The structure of raft domains in the plasma membrane of splenocytes from untreated and glycosphingolipid-treated mice was analyzed in order to determine whether administration of glycosphingolipids affects lipid raft disruption on the splenic plasma membrane. Fluorescence microscopy for CTx-FITC staining was utilized to examine GM1 clustering patterns. Equal numbers of cells (5×10^6) were used for the GM1 clustering assay. Representative images are presented in **Fig. 6**. Administration of TNBS led to a mild induction of raft clustering by CTx. Mice treated with GC and IGL showed enlarged GM1 patches on the cell surface, suggesting an increase in the GM1 content on the

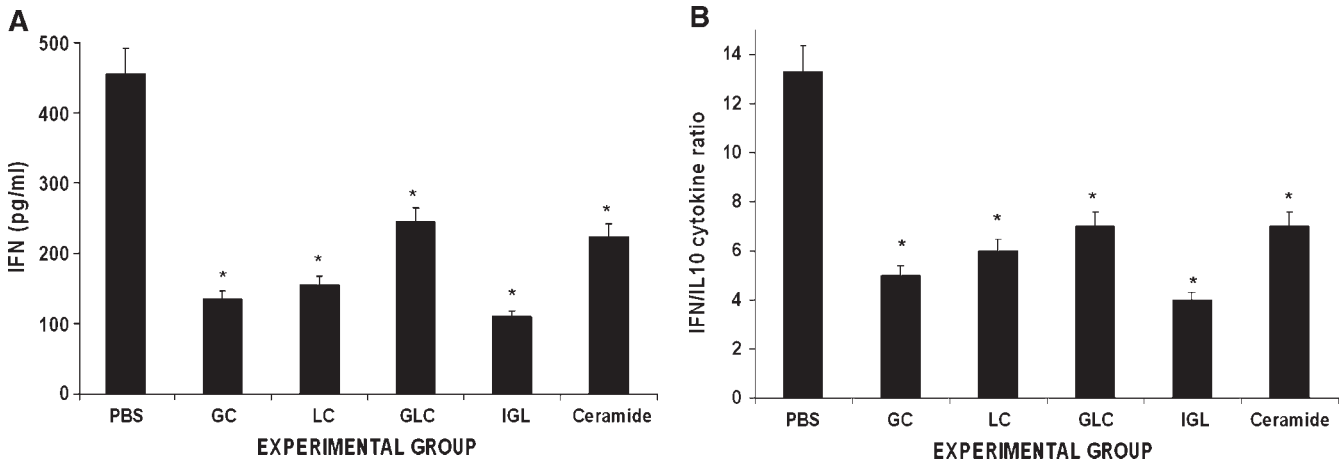

Fig. 3. Effects of β-glycosphingolipids on serum cytokine levels. A: Serum interferon-γ (IFN-γ) levels were measured by ELISA. B: Calculating the IFN-γ/interleukin-10 (IL-10) serum cytokine ratio reveals that administration of GC and IGL led to a significant reduction in the IFN-γ/IL-10 ratio. * $P < 0.05$.

membranes of splenocytes. No significant changes were noted in naïve animals.

Effect of β-glycosphingolipids on the microscopic colitis score and extent of disease

Administration of GC and IGL was associated with a significant improvement in the microscopic colitis score. The total score decreased from 5.6 for mice in group A to 3.9 and 3.5 for mice in groups B and E, respectively ($P < 0.005$; **Fig. 7A**). Similarly, the extent of bowel affected by the disease was reduced significantly in animals treated with GC and IGL (0.85 and 0.75 for mice in groups B and E, respectively, vs. 1.9 in group A; $P < 0.005$; Fig. 7B). Representative histological slides are shown in Fig. 7C.

DISCUSSION

Administration of β-glycosphingolipids significantly altered both the distribution of NKT cells and the intrahepatic $CD8^+$ lymphocyte trapping in mice with experimentally induced colitis. Changes in lipid rafts were noted in $CD8^+$ and $CD4^+$ lymphocytes in the affected animals. The immune modulatory effects led to a shift in the Th1/Th2 immune paradigm. By comparison, these ligands had no effect in naïve animals, suggesting that the immune modulatory effect depends on signals elicited by the microenvironment.

The induction of peripheral tolerance via oral administration of an antigen or FK506 treatment was associated with an increase in the proportion of intrahepatic NKT

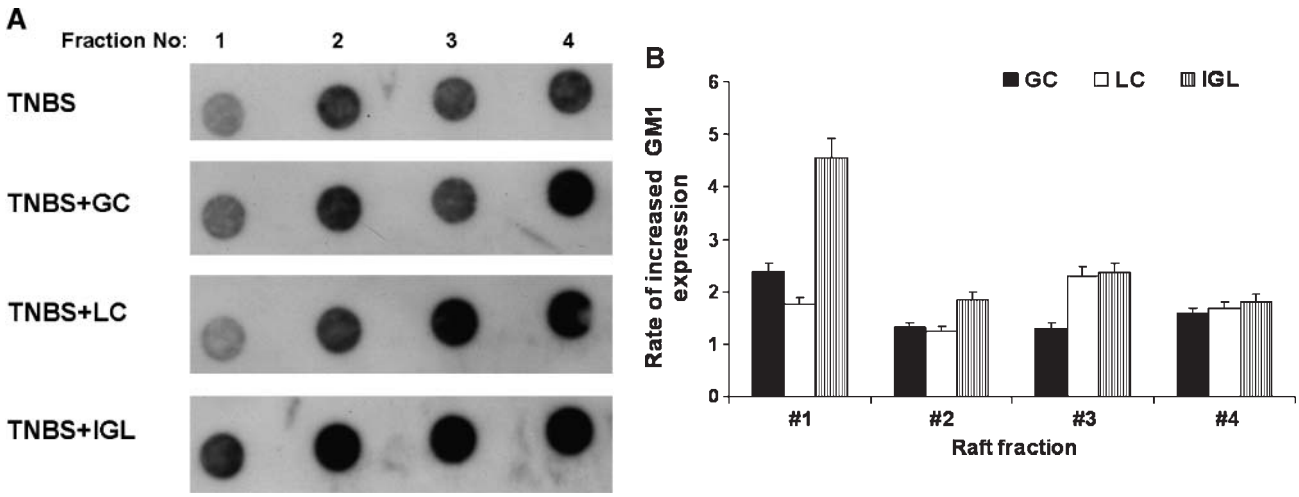

Fig. 4. Immunoblotting characterization of lipid microdomain fractions. A: Identification of GM1 distribution in the detergent-insoluble fractions was analyzed by dot blot analysis. Quantification of the data was performed using densitometry of the blot. In trinitrobenzene sulfonic acid (TNBS)-treated mice, administration of GC, LC, and IGL resulted in alteration of both the lipid raft and cytosolic fractions. In contrast, administration of β-glycosphingolipids was not associated with a significant change in GM1 content in naïve mice. B: For fractions 1, 2, 3, and 4, administration of GC, LC, and IGL increased the GM1 by 2.37, 1.76, 4.55; 1.32, 1.25, 1.85; 1.31, 2.29, 2.35; and 1.58, 1.68, 1.81, respectively. Error bars indicate ± SD.

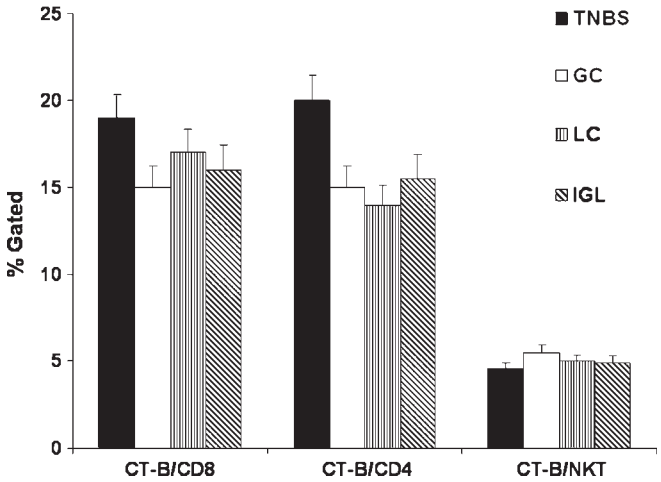

Fig. 5. Effects of β-glycosphingolipids on lipid rafts and cell membranes. FACS analysis of cholera toxin (CTx)-FITC staining was performed to determine GM1 expression on the cell surface in lymphocyte subsets ($CD4^+$, $CD8^+$, and NKT cells). Administration of TNBS led to a major decrease of GM1 expression in $CD4^+$, $CD8^+$, and NKT cells. Administration of GC, LC, and IGL was associated with an additional decrease in GM1 expression on $CD8^+$ and $CD4^+$ lymphocytes but not on NKT cells. Error bars indicate ± SD.

cells and an increase in cytotoxicity (10, 31). In the present study, both GC and IGL were associated with a significant increase in the number of intrahepatic NKT lymphocytes, whereas administration of LC, GLC, and ceramide was associated with a decrease in intrahepatic NKT cell number.

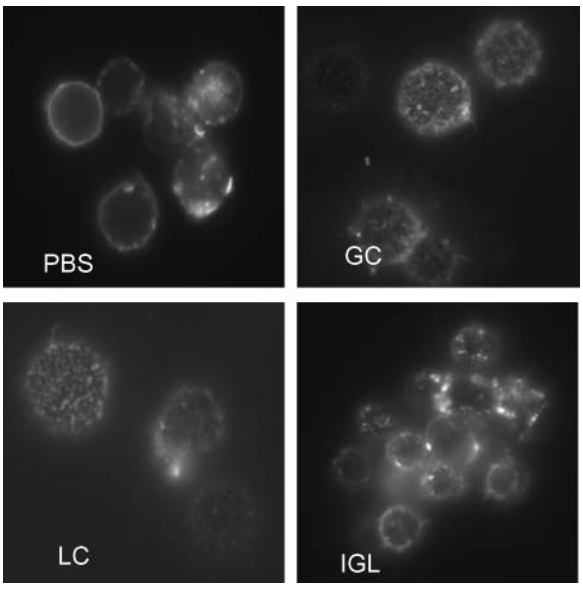

Fig. 6. Fluorescence microscopy with CTx. Fluorescence microscopy for CTx-FITC staining was utilized to examine GM1 clustering patterns. Equal numbers of cells (5×10^6) were used for the GM1 clustering assay. Representative images of the clustering patterns of GM1 are shown using fluorescence microscopy of CTx-FITC staining. Administration of TNBS led to a mild induction of raft clustering by CTx. Mice treated with GC and IGL showed enlarged GM1 patches on the cell surface, suggesting an increase in the GM1 content on the membranes of splenocytes.

The intrahepatic-to-intrasplenic NKT ratio was increased significantly for mice treated with GC and IGL. Interestingly, no significant effect was noted with respect to the peripheral NKT cell number. β- Glycosphingolipid-dependent alteration of the intrahepatic NKT distribution was observed in mice with experimentally induced colitis but not in naïve animals. These data clearly indicate that the environment in which NKT lymphocytes become activated, the different types of stimuli, and the signaling receptors all play a role in determining the function of these cells (1, 29, 32). Furthermore, results presented here reveal that signals from the immune environment are important in determining the β-glycosphingolipid-mediated redistribution of NKT cells.

Administration of GC and IGL was associated with a significant increase in intrahepatic $CD8^+$ lymphocyte trapping, in contrast to the marked decrease in intrahepatic $CD8^+$ lymphocyte trapping induced by colitis. In addition, the administration of both GC and IGL was associated with a significant reduction in the IFN-γ/IL-10 ratio and alleviation of colitis. We speculate that glycosphingolipids may suppress a T cell clone that normally undergoes expansion during colitis. The intrahepatic sequestration of activated $CD8^+$ T cells suggests a primary liver event, although this may be dependent on the intrahepatic function of NKT cells as manifested by the selective increase in the liver during tolerance induction. Intrahepatic T cell deletion is one of the mechanisms by which the liver induces tolerance. This type of tolerance is explained by an active trapping (and possibly killing) mechanism that causes apoptosis in intrahepatic T cells. Activation of $CD8^+$ T cells in the periphery is accompanied by lymphocyte trafficking to the liver (1, 28). The liver is a site for antigen-specific trapping of T cells during activation-mediated cell death (33) and serves as a site for apoptosis of $CD8^+$ T cells in models of diabetes, influenza virus infection, and lymphopenia (30–32). The preferential trapping of $CD8^+$ T cells in the liver may be a consequence of local presentation of antigenic peptide by MHC class I molecules that are expressed in the liver, or may be due to an adhesion-based property of the $CD8^+$ T cell subset (34). The results of the present study suggest that $CD8^+$ T lymphocytes are trapped in the liver as part of a general mechanism of termination of an immune response. $CD8^+$ T lymphocyte trapping correlated with the clinical status of experimental colitis. The effect of the $CD8^+$ lymphocyte redistribution on the shift from pro- to anti-inflammatory function was evident in changes in the IFN-γ/IL-10 cytokine balance.

The immune modulatory effect of β-glycosphingolipids was associated with alteration of lipid rafts on lymphocyte membranes. Raft microdomains are enriched in the ganglioside GM1, which is recognized by CTx and serves as a raft marker (35). Dot blot analysis of GM1 content in different cell membrane fractions showed that administration of GC, LC, and IGL to TNBS-treated mice led to alterations in the GM1 content of the raft fractions. FACS analysis of the expression of GM1 on the cell surface revealed that treatment with GC, LC, and IGL significantly decreased GM1 expression. These effects were observed mainly on the surface of $CD8^+$ and $CD4^+$ T lymphocytes

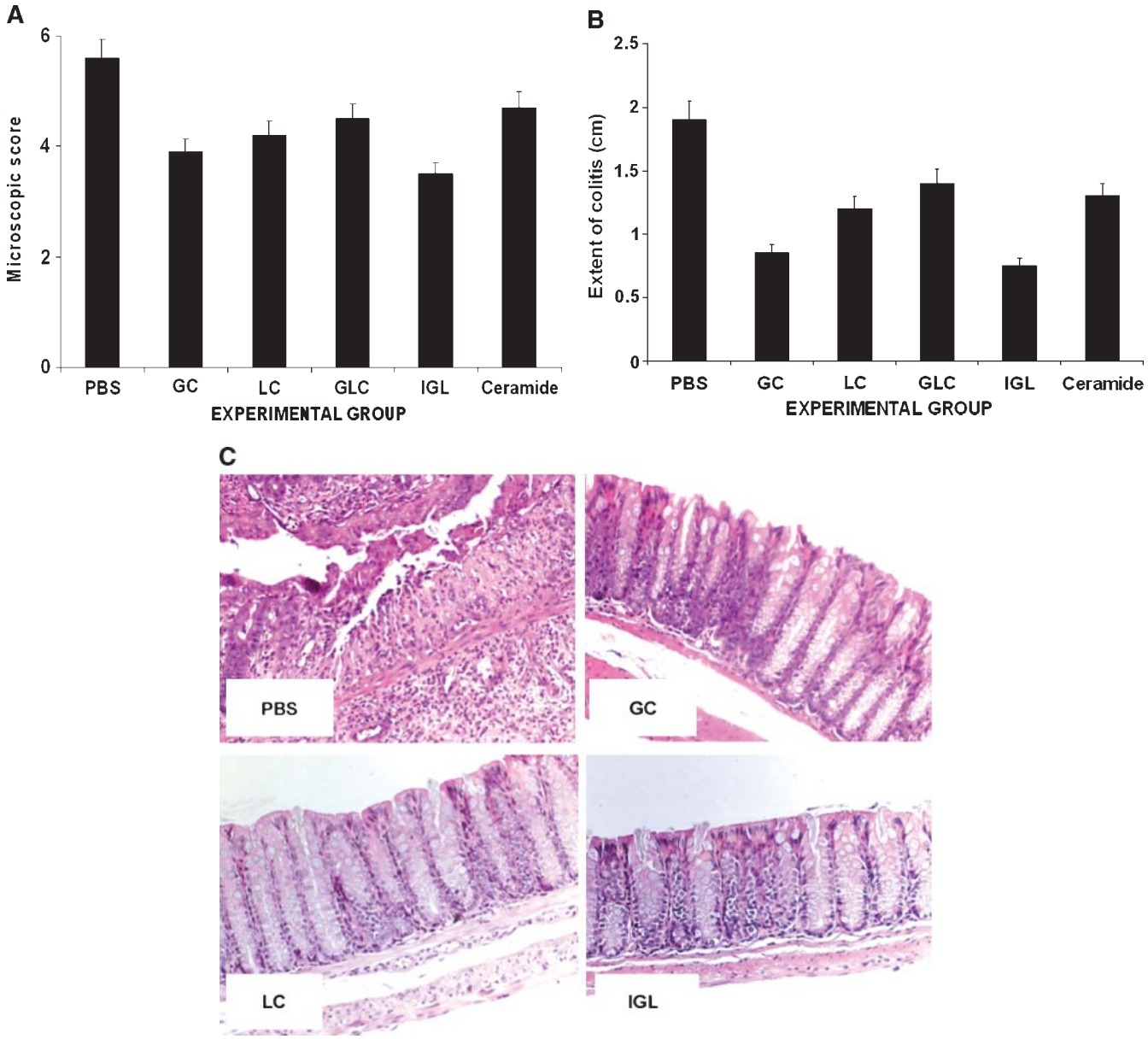

Fig. 7. Effect of β-glycosphingolipids on colitis. A: Five paraffin sections from each mouse (treated and control) were stained with hematoxylin-eosin using standard techniques. The degree of inflammation on microscopic cross sections of the colon was graded semi-quantitatively from 0 to 6. Administration of GC and IGL was associated with a significant improvement in the microscopic colitis score. B: The extent of disease was assessed by evaluation of the entire bowel taken from each animal. The region of the bowel affected by the disease was reduced significantly in animals treated with GC and IGL. Error bars indicate ± SD. C: Representative hematoxylin-eosin-stained slides from the bowels of treated and control animals (×10) show a decrease in inflammation and preserved bowel architecture in GC- and IGL-treated animals.

and not on NKT cells. This specificity may be related to changes observed in the $CD4^+/CD8^+$ ratio as a consequence of TNBS administration, which altered the relevant signaling pathway. Activated Th1 and Th2 cells have distinct patterns of membrane compartmentalization into lipid rafts. Components of membrane microdomains are differentially regulated in functionally distinct $CD4^+$ T cells (36). CTx and actin colocalize to the same membrane microdomains, suggesting a possible functional association (37). Lipid rafts are highly dynamic, submicroscopic assemblies that float freely within the disordered liquid bilayer in cell membranes. These rafts can coalesce upon clustering of their components. The clustering of small individual rafts together into larger visible units facilitates efficient interaction of raft-associated proteins. The data from the present study indicate that in mice with colitis, clustering patterns of GM1 were associated with a mild induction of raft clustering. Following treatment with GC and IGL, these mice showed enlarged GM1 patches on the cell surface of splenocytes, suggesting an increase in the clustering effect of these ligands.

Administration of β-glycosphingolipids may affect lipid raft composition, via a direct effect. This may involve the incorporation of glycosphingolipid between the two membrane

leaflets, leading to budding/fusion processes, blebbing, and morphological changes in the plasma and mitochondrial membranes (38, 39). Alteration of lipid rafts may affect the intracellular machinery as a means of immune modulation. Modification of the conformation of proteins involved in cell signaling can occur as a consequence of alteration of membrane properties, and this alteration may lead to either their activation or their inhibition (40, 41). Administration of exogenous gangliosides to cells promotes their incorporation into the rafts (38) and may lead to alterations in membrane integrity and, as a result, downstream signaling (39, 40). Other types of alterations have been reported in raft distribution, composition, and dynamics in immune-mediated disorders (41). T cells in systemic lupus erythematosus have a more extensive lipid raft pool and are more robust in their capacity to generate lipid rafts (17). The β-glycosphingolipid effects on lipid rafts were observed mainly in diseased animals and not in naïve mice, suggesting that lymphocyte membrane dysfunction is associated with the development of the disease state. In addition, these ligands may exert fine tuning or adjustment of disrupted rafts. Alternatively, alteration of lipid rafts by β-glycosphingolipid may involve signals that are present only in a diseased state.

The GC- and IGL-mediated alterations in NKT lymphocyte distribution, $CD8^+$ trapping, and lipid raft formation were associated with a significant improvement in the microscopic colitis score and a reduction in the extent of bowel disease. Several mechanisms may explain these effects. Previous reports implicated β-glycosphingolipids in the alteration of the plasticity of NKT regulatory cells in opposing immune environments (8, 42). Alternatively, β-glycosphingolipids may affect NKT cells directly or indirectly via an effect on dendritic cells that would lead to peripheral migration. Both possibilities are dependent on the effects of β-glycosphingolipids on lipid rafts. The differential effects of the various β-glycosphingolipids used in the present study and the potentiation of the immune modulatory effect noted in mice treated with IGL may be associated with the importance of the β structure and the sugar moiety structure in receptor binding. Alternatively, the different ligands may depend on different signals from the environment. In addition, the present study does not exclude the possibility of a non-CD1d-dependent effect of β-glycosphingolipids. Such an effect is supported by the effect of glycosphingolipids on the alteration of lipid rafts, mainly on $CD4^+$ and $CD8^+$ T cells. Although this result may represent a secondary effect on CD1d-expressing cells, these ligands may exert an immune modulatory effect via alteration of the immunological synapse or the intracellular machinery of effector cells. The effect on NKT lymphocyte distribution may be secondary to the alteration of intrahepatic signaling via $CD8^+$ lymphocytes.

In summary, administration of β-glycosphingolipids altered the intrahepatic redistribution of NKT, $CD4^+$, and $CD8^+$ lymphocytes and altered the composition of lipid rafts on these cells in an environmentally dependent signaling manner. These data clearly support the notion that alteration of plasma membranes using physiological doses of β-glycosphingolipids may provide a novel therapeutic approach for the alteration of the immunological synapse.

REFERENCES

1. Crispe, I. N., T. Dao, K. Klugewitz, W. Z. Mehal, and D. P. Metz. 2000. The liver as a site of T-cell apoptosis: graveyard, or killing field? *Immunol. Rev.* **174:** 47–62.
2. Mehal, W. Z., A. E. Juedes, and I. N. Crispe. 1999. Selective retention of activated CD8+ T cells by the normal liver. *J. Immunol.* **163:** 3202–3210.
3. Crispe, I. N., M. Giannandrea, I. Klein, B. John, B. Sampson, and S. Wuensch. 2006. Cellular and molecular mechanisms of liver tolerance. *Immunol. Rev.* **213:** 101–118.
4. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nat. Rev. Immunol.* **4:** 231–237.
5. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu. Rev. Immunol.* **25:** 297–336.
6. Racanelli, V., and B. Rehermann. 2006. The liver as an immunological organ. *Hepatology.* **43 (Suppl.):** 54–62.
7. Brutkiewicz, R. R. 2006. CD1d ligands: the good, the bad, and the ugly. *J. Immunol.* **177:** 769–775.
8. Zigmond, E., S. Preston, O. Pappo, G. Lalazar, M. Margalit, Z. Shalev, L. Zolotarov, D. Friedman, R. Alper, and Y. Ilan. 2007. Beta-glucosylceramide: a novel method for enhancement of natural killer T lymphoycte plasticity in murine models of immune-mediated disorders. *Gut.* **56:** 82–89.
9. Shibolet, O., R. Alper, L. Zlotogarov, B. Thalenfeld, D. Engelhardt, E. Rabbani, and Y. Ilan. 2003. NKT and CD8 lymphocytes mediate suppression of hepatocellular carcinoma growth via tumor antigen-pulsed dendritic cells. *Int. J. Cancer.* **106:** 236–243.
10. Shibolet, O., R. Alper, L. Zolotarov, S. Trop, B. Thalenfeld, D. Engelhardt, E. Rabbani, and Y. Ilan. 2004. The role of intrahepatic CD8+ T cell trapping and NK1.1+ cells in liver-mediated immune regulation. *Clin. Immunol.* **111:** 82–92.
11. Stanic, A. K., J. J. Park, and S. Joyce. 2003. Innate self recognition by an invariant, rearranged T-cell receptor and its immune consequences. *Immunology.* **109:** 171–184.
12. Tsuji, M. 2006. Glycolipids and phospholipids as natural CD1d-binding NKT cell ligands. *Cell. Mol. Life Sci.* **63:** 1889–1898.
13. Van Kaer, L. 2005. alpha-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat. Rev. Immunol.* **5:** 31–42.
14. Zhou, D., J. Mattner, C. Cantu 3rd, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y. P. Wu, T. Yamashita, et al. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science.* **306:** 1786–1789.
15. Zhou, D. 2006. The immunological function of iGb3. *Curr. Protein Pept. Sci.* **7:** 325–333.
16. Porubsky, S., A. O. Speak, B. Luckow, V. Cerundolo, F. M. Platt, and H. J. Grone. 2007. Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. *Proc. Natl. Acad. Sci. USA.* **104:** 5977–5982.
17. Sriram, V., W. Du, J. Gervay-Hague, and R. R. Brutkiewicz. 2005. Cell wall glycosphingolipids of Sphingomonas paucimobilis are CD1d-specific ligands for NKT cells. *Eur. J. Immunol.* **35:** 1692–1701.
18. Brutkiewicz, R. R., Y. Lin, S. Cho, Y. K. Hwang, V. Sriram, and T. J. Roberts. 2003. CD1d-mediated antigen presentation to natural killer T (NKT) cells. *Crit. Rev. Immunol.* **23:** 403–419.
19. Stanic, A. K., A. D. De Silva, J. J. Park, V. Sriram, S. Ichikawa, Y. Hirabyashi, K. Hayakawa, L. Van Kaer, R. R. Brutkiewicz, and S. Joyce. 2003. Defective presentation of the CD1d1-restricted natural Va14Ja18 NKT lymphocyte antigen caused by beta-D-glucosylceramide synthase deficiency. *Proc. Natl. Acad. Sci. USA.* **100:** 1849–1854.
20. Ortaldo, J. R., H. A. Young, R. T. Winkler-Pickett, E. W. Bere, Jr., W. J. Murphy, and R. H. Wiltrout. 2004. Dissociation of NKT stimulation, cytokine induction, and NK activation in vivo by the use of distinct TCR-binding ceramides. *J. Immunol.* **172:** 943–953.
21. Margalit, M., S. A. Ghazala, R. Alper, E. Elinav, A. Klein, V. Doviner, Y. Sherman, B. Thalenfeld, D. Engelhardt, E. Rabbani, et al. 2005. Glucocerebroside treatment ameliorates ConA hepatitis by inhibition of NKT lymphocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **289:** G917–G925.
22. Margalit, M., Z. Shalev, O. Pappo, M. Sklair-Levy, R. Alper, M. Gomori, D. Engelhardt, E. Rabbani, and Y. Ilan. 2006. Glucocerebroside ame-

liorates the metabolic syndrome in OB/OB mice. *J. Pharmacol. Exp. Ther.* **319:** 105–110.
23. Helms, J. B., and C. Zurzolo. 2004. Lipids as targeting signals: lipid rafts and intracellular trafficking. *Traffic.* **5:** 247–254.
24. Anderson, H. A., E. M. Hiltbold, and P. A. Roche. 2000. Concentration of MHC class II molecules in lipid rafts facilitates antigen presentation. *Nat. Immunol.* **1:** 156–162.
25. Brown, D. A., and E. London. 2000. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* **275:** 17221–17224.
26. Park, Y. K., J. W. Lee, Y. G. Ko, S. Hong, and S. H. Park. 2005. Lipid rafts are required for efficient signal transduction by CD1d. *Biochem. Biophys. Res. Commun.* **327:** 1143–1154.
27. Yu, K. O., J. S. Im, A. Molano, Y. Dutronc, P. A. Illarionov, C. Forestier, N. Fujiwara, I. Arias, S. Miyake, T. Yamamura, et al. 2005. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc. Natl. Acad. Sci. USA.* **102:** 3383–3388.
28. Ilan, Y., S. Weksler-Zangen, S. Ben-Horin, J. Diment, B. Sauter, E. Rabbani, D. Engelhardt, N. R. Chowdhury, J. R. Chowdhury, and E. Goldin. 2000. Treatment of experimental colitis by oral tolerance induction: a central role for suppressor lymphocytes. *Am. J. Gastroenterol.* **95:** 966–973.
29. Trop, S., D. Samsonov, I. Gotsman, R. Alper, J. Diment, and Y. Ilan. 1999. Liver-associated lymphocytes expressing NK1.1 are essential for oral immune tolerance induction in a murine model. *Hepatology.* **29:** 746–755.
30. Rouvinski, A., I. Gahali-Sass, I. Stav, E. Metzer, H. Atlan, and A. Taraboulos. 2003. Both raft- and non-raft proteins associate with CHAPS-insoluble complexes: some APP in large complexes. *Biochem. Biophys. Res. Commun.* **308:** 750–758.
31. Samsonov, D., R. Alper, and Y. Ilan. 2001. Pregnancy rate and embryo loss in the NK1.1+ T cell-depleted mouse. *J. Reprod. Med.* **46:** 54–60.
32. Hammond, K. J., and D. I. Godfrey. 2002. NKT cells: potential targets for autoimmune disease therapy? *Tissue Antigens.* **59:** 353–363.
33. Renno, T., M. Hahne, J. Tschopp, and H. R. MacDonald. 1996. Peripheral T cells undergoing superantigen-induced apoptosis in vivo express B220 and upregulate Fas and Fas ligand. *J. Exp. Med.* **183:** 431–437.
34. Bonfoco, E., P. M. Stuart, T. Brunner, T. Lin, T. S. Griffith, Y. Gao, H. Nakajima, P. A. Henkart, T. A. Ferguson, and D. R. Green. 1998. Inducible nonlymphoid expression of Fas ligand is responsible for superantigen-induced peripheral deletion of T cells. *Immunity.* **9:** 711–720.
35. Thomas, S., A. Preda-Pais, S. Casares, and T. D. Brumeanu. 2004. Analysis of lipid rafts in T cells. *Mol. Immunol.* **41:** 399–409.
36. Balamuth, F., D. Leitenberg, J. Unternaehrer, I. Mellman, and K. Bottomly. 2001. Distinct patterns of membrane microdomain partitioning in Th1 and th2 cells. *Immunity.* **15:** 729–738.
37. Badizadegan, K., H. E. Wheeler, Y. Fujinaga, and W. I. Lencer. 2004. Trafficking of cholera toxin-ganglioside GM1 complex into Golgi and induction of toxicity depend on actin cytoskeleton. *Am. J. Physiol.* **287:** C1453–C1462.
38. Simons, M., T. Friedrichson, J. B. Schulz, M. Pitto, M. Masserini, and T. V. Kurzchalia. 1999. Exogenous administration of gangliosides displaces GPI-anchored proteins from lipid microdomains in living cells. *Mol. Biol. Cell.* **10:** 3187–3196.
39. Janes, P. W., S. C. Ley, and A. I. Magee. 1999. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J. Cell Biol.* **147:** 447–461.
40. Rodgers, W., B. Crise, and J. K. Rose. 1994. Signals determining protein tyrosine kinase and glycosyl-phosphatidylinositol-anchored protein targeting to a glycolipid-enriched membrane fraction. *Mol. Cell. Biol.* **14:** 5384–5391.
41. Krishnan, S., M. P. Nambiar, V. G. Warke, C. U. Fisher, J. Mitchell, N. Delaney, and G. C. Tsokos. 2004. Alterations in lipid raft composition and dynamics contribute to abnormal T cell responses in systemic lupus erythematosus. *J. Immunol.* **172:** 7821–7831.
42. Godfrey, D. I., and M. Kronenberg. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest.* **114:** 1379–1388.