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Glucocerebroside treatment ameliorates ConA hepatitis by inhibition of NKT lymphocytes

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Margalit, Maya, Samir Abu Ghazala, Ruslana Alper, Eran Elinav, Athalia Klein, Victoria Doviner, Yoav Sherman, Barbara Thalenfeld, Dean Engelhardt, Elazar Rabbani, and Yaron Ilan. Glucocerebroside treatment ameliorates ConA hepatitis by inhibition of NKT lymphocytes. *Am J Physiol Gastrointest Liver Physiol* 289: G917–G925, 2005. First published June 23, 2005; doi:10.1152/ajpgi.00105.2005.—Concanavalin A (ConA) induces natural killer T (NKT) cell-mediated liver damage. Glucocerebroside (GC) is a naturally occurring glycolipid. Our aims were to determine the effect of GC in a murine model of ConA-induced hepatitis. Mice in *groups A* and *B* were treated with GC 2 h before and 2 h following administration of ConA, respectively; *group C* mice were treated with ConA; *group D* mice was treated with GC; *group E* mice did not receive any treatment. Liver damage was evaluated by serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and liver histology. The immune effect of GC was determined by fluorescence-activated cell sorter analysis of intrahepatic and intrasplenic NKT lymphocytes, measurement of cytokine levels, and Western blot analysis for STAT 1, 4, 6, and NF-κB expression. The effect of GC on NKT cell proliferation was assessed in vitro. Serum AST and ALT levels were markedly reduced in GC-treated *group A* mice compared with nontreated *group C* animals, and histological damage was markedly attenuated in *group A*. The beneficial effect of GC was associated with a 20% decrease of intrahepatic NKT lymphocytes, significant lowering of serum IFN-γ levels, and decreased STAT1 and STAT6 expression. In vitro administration of GC led to a 42% decrease of NKT cell proliferation in the presence of dendritic cells but not in their absence. Intraperitoneally administered radioactive GC was detected in the liver and bowel. Administration of GC led to amelioration of ConA hepatitis associated with an inhibitory effect on NKT lymphocytes. GC holds promise as a new immune-modulatory agent.

glycolipid

NATURAL KILLER T (NKT) lymphocytes are a subset of regulatory lymphocytes that coexpress cell surface receptor characteristics of both T lymphocytes (e.g., CD3, α/β-T cell receptor) and natural killer cells (e.g., NK1.1) (1). In mice, most NKT cells express the invariant Vα14Jα281 TCR chain, paired with a limited number of β-chain types (Vβ8.2, Vβ7, and Vβ2). In humans, distinct populations of NKT cells express the homologous invariant Vα24 paired with Vβ11 (2). NKT lymphocytes are activated by interaction of their TCR with glycolipids presented by CD1d, a nonpolymorphic, MHC class I-like molecule expressed by antigen presenting cells. CD1d is also expressed by hepatocytes (3). A possible ligand for NKT cells was recently suggested (4). α-Galactosylceramide (α-GalCer; KRN-7000), a sponge-derived glycolipid, is a potent activator of both mouse and human NKT cells (5). Administration of α-GalCer leads to rapid production of both IFN-γ and IL-4 by NKT cells, with secondary activation of innate and adaptive immune responses (6, 7). NKT cells also recognize glycosylphosphatidylinositol (GPI) anchors of *Plasmodium, Trypanosoma* and *Leishmania*, and phosphatidylinositol-mannosides derived from *Mycobacterium tuberculosis* (1)

Activation of NKT lymphocytes can lead to significant liver damage (8, 9). Concanavalin A (ConA), a plant lectin and T cell mitogen, rapidly induces severe immune-mediated hepatitis in mice that is associated with increased TNF-α, IFN-γ, IL-12, IL-18, and IL-4 expression (10) and in which NKT lymphocytes, CD4+ T cells, and Kupffer cells have a contributory role (11). Vα14 NKT cells were shown to be required and sufficient for induction of this type of liver injury (12). The cytotoxic activity of NKT lymphocytes is augmented by autocrine secretion of IL-4, leading to increased expression of granzyme B and Fas ligand by NKT lymphocytes; Vα14 NKT cells from perforin knockout or FasL-mutant gld/gld mice fail to induce hepatitis. In another study, adoptive transfer of NKT cells from wild-type, but not from FasL-deficient gld mice, sensitized CD1-deficient mice, which lack NKT cells, to ConA-induced hepatitis (13). NKT cells also have a central role in LPS, α-GalCer, and salmonella infection-induced liver injury (14–16) in hepatic injury secondary to deletion of suppressor of cytokine signaling-1 (17) and in hepatic damage in the setting of chronic hepatitis C infection and primary biliary cirrhosis (18, 19).

Glucocerebroside (β-glucosylceramide; GC), a naturally occurring glycolipid, is a metabolic intermediate in the anabolic and catabolic pathways of complex glycosphingolipids (20). Its synthesis from ceramide is catalyzed by the enzyme glucosylceramide synthase. Overexpression of glucosylceramide synthase characterizes a number of MDR tumor cell lines (21). The MDR phenotype was postulated to result from altered levels of ceramide (22) and gangliosides (23); whereas the former is known to exert a proapoptotic effect, the latter may promote tumorigenesis by enabling tumor cells to evade immunosurveillance. Inherited deficiency of glucocerebrosidase, a lysosomal hydrolase, results in Gaucher's disease (24). Patients with Gaucher's disease have altered humoral and cellular immune profiles (25, 26) and increased peripheral blood NKT lymphocytes (27).

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The effect of glucocerebroside on NKT cells is not known. CD1d-bound glucocerebroside does not activate NKT cells directly and may inhibit activation of NKT cells by α-GalCer (5, 28). On the other hand, glucosylceramide-synthase deficiency was shown to lead to defective ligand presentation by CD1d, with secondary inhibition of NKT cell activation (28).

The aim of the present study was to determine the immune modulatory effect of GC in a murine model of ConA hepatitis. In vitro, administration of GC induced a dendritic cell-dependent decrease of NKT cell proliferation; in vivo, significant amelioration of ConA hepatitis was observed.

METHODS

Preparation of Glycolipids

β-Glucosylceramide was purchased from Avanti Polar Lipids (Alabaster, AL; Catalogue #131304), dissolved in ethanol, and emulsified in PBS.

In Vitro Effect of GC

NKT lymphocytes and dendritic cells were isolated from spleens of naïve mice. Harvested NKT lymphoyctes were placed in 24-well plates. Naïve NKT lymphocytes (1×10^6/well) were incubated for 24 h in medium with or without glucocerebroside (100 ng/ml) in the presence or absence of dendritic cells (1×10^4/well). Data are presented as mean stimulation indexes (SI) of triplicates, calculated from the ratios of incorporated radioactivities in the presence or absence of antigen.

Radiolabeled GC

To determine the tissue distribution of GC, four groups of mice (*A-D*, $n = 2$ per group) were administered radiolabeled GC (Avanti Polar Lipids, Alabaster, AL) orally (10 μg/mouse, *groups A* and *C*) or by a single intraperitoneal injection (1 μg/mouse, *groups B* and *D*). *Groups A* and *B* animals were killed after 2 h; *groups C* and *D* animals were killed after 48 h. Brain, liver, heart, lung, kidney, spleen, small intestine, and colon were removed and homogenized in double-distilled water. Radioactivity per 100 mg tissue, expressed as counts per minute (cpm), was determined by a liquid scintillation analyzer (Opti-Flour, Packard, MA).

Animals

Eight-week-old male BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal core of the Hadassah-Hebrew University Medical School. Mice were administered standard laboratory chow and water ad libitum and kept in 12:12-h light-dark cycles. Animal experiments were carried out according to the guidelines of the Hebrew University-Hadassah Institutional Committee for Care and Use of Laboratory Animals and with the committee's approval.

Induction of ConA Hepatitis

ConA (Sigma) was dissolved in pyrogen-free PBS and injected into the tail vein at a dose of 500 μg/mouse (~15 mg/kg).

Experimental Groups

Three ConA-injected groups (*A-C*) and two noninjected groups (*D* and *E*) of BALB/c mice, 12 mice per group, were studied (Table 1). *Group A* mice were administered a single intraperitoneal injection of glucocerebroside (1 μg GC in 100 μl PBS) 2 h before intravenous administration of ConA. *Group B* mice were similarly injected with GC 2 h after intravenous administration of ConA. *Group C* mice were administered ConA. *Group D* mice were treated with GC. *Group E* mice were not administered ConA or GC. *Group B-E* animals were killed after 8 h; *group A* animals were killed after 10 h (8 h after injection of ConA). For all animals, determination of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, histological examination of liver specimens, FACS analysis of intrahepatic and intrasplenic lymphocytes for NKT markers, measurement of serum cytokine levels, and Western blot analysis for the expression of the transcriptoin factors STAT 1, 4, and 6 and NF-κB were performed.

Table 1. *Experimental groups*

Group	ConA	GC
A	+	+ (2 h before ConA)
B	+	+ (2 h after ConA)
C	+	−
D	−	+
E	−	−

ConA, Concanavalin A; GC, glucocerebroside.

Liver Enzymes

Sera from individual mice were obtained. Serum AST and ALT levels were measured by an automatic analyzer.

Histological Examination

Hematoxylin/eosin staining of paraffin-embedded liver sections was performed. Sections were examined by two experienced pathologists (V. Doviner, Y. Sherman) that were blinded to the experiment conditions.

Cytokine Measurement

Serum IFN-γ, IL-2, IL-12, IL-4, and IL-10 levels were measured in each animal by "sandwich" ELISA, using commercial kits (Genzyme Diagnostics, Boston, MA).

Isolation of Splenocytes and Intrahepatic Lymphocytes

Splenocytes and intrahepatic lymphocytes were isolated as previously described (29). In brief, after flushing of the liver with cold PBS and removal of connective tissue, livers and spleens were crushed through a stainless mesh (size 60, Sigma, St. Louis, MO). Cell suspensions in PBS were centrifuged (1,250 rpm for 10 min) for removal of debris and placed through a nylon mesh presoaked in PBS. Unbound cells were collected, and 20 ml of histopague 1077 (Sigma Diagnostics, St. Louis, MO) were slowly placed under the cells; after centrifugation (1,640 rpm for 15 min at room temperature), cells at the interface were collected and washed twice. Approximately 1×10^6 cells/mouse liver were recovered. Viability was determined to be >95% by trypan blue staining.

NKT Cell Proliferation Assays

NKT cells were grown in triplicates of 10^5 cells in RPMI with 10% FCS and were stimulated in vitro using 1 μg/ml of GC, in the presence or absence of dendritic cells (1:1 ratio). Forty eight hours later, [methyl-H^3]thymidine was added (1 μCi/ml; Amersham Pharmacia, Biotech). NKT-cell cultures were harvested following 12 h. Controls were incubated in the presence of 2.5 μg/ml phytohemagglutinin or medium alone. Data were given as mean SI of triplicates ± SE, calculated from the ratios of incorporated radioactivities of lymphocyte cultures expressed as counts per minute.

STAT Protein Expression

Expression of the transcription factors signal transducer and activator of transcription (STAT) 1, 4, and 6 and NF-κB in splenocytes

was determined by Western blot analysis of splenocytes harvested from mice in *groups A-D*. Splenocytes (10×10^6) were lysed in 100 μl of lysis solution (Sigma). Proteins (100 μg/lane) were resolved by electrophoresis on SDS-polyacrylamide (7.5%) gels and electroblotted to nitrocellulose membranes (Schleicher & Scuell). Probing with a polyclonal rabbit anti-mouse antibody for the different tested STAT proteins and NF-κB (Santa Cruz Biothechnology) was followed by addition of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno Research).

FACS Analysis for Determination of NKT Lymphocyte Percentage

Immediately after lymphocyte isolation, triplicates of $2–5 \times 10^4$ cells/500 μl PBS were placed into Falcon 2052 tubes, incubated with 4 ml of 1% BSA for 10 min, and centrifuged at 350 *g* for 5 min. For determination of the percentage of NKT lymphocytes, anti-CD3 and anti DX5 antibodies were used (Pharmingen). Analytical cell sorting was performed on 1×10^4 cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson, Oxnard, CA). Only live cells were counted, and background fluorescence from nonantibody-treated lymphocytes was subtracted. 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.

Isolation of NKT Lymphocytes and Dendritic Cells

Cell separation was performed using magnetic cell sorting (MACS, Miltenyi Biotec) according to the manufacturer's instructions. Anti-CD3 and anti-DX5 magnetic beads were used for separation of NKT lymphocytes; anti-CD11c beads served for separation of dendritic cells. Beads were removed between the two steps according to the manufacturer's instructions. Above 95% accuracy was achieved by FACS analysis of cells.

Statistical Analysis

Statistical analysis was performed using the Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Effect of GC on NKT Cell Proliferation in vitro

The effect of incubation with GC on NKT cell proliferation was examined in vitro (Fig. 1). Incubation of NKT lymphocytes with glucocerebroside did not affect NKT cell proliferation significantly (SI 1.2 vs. 1.13 in the absence and presence of glucocerebroside, respectively). In contrast, in the presence of dendritic cells, NKT cell proliferation was markedly inhibited by glucocerebroside (SI 0.44). This finding supports a role for antigen presentation in the inhibitory effect of glucocerebroside. Interestingly, the mere presence of dendritic cells was also found to inhibit NKT cell proliferation (SI 0.76).

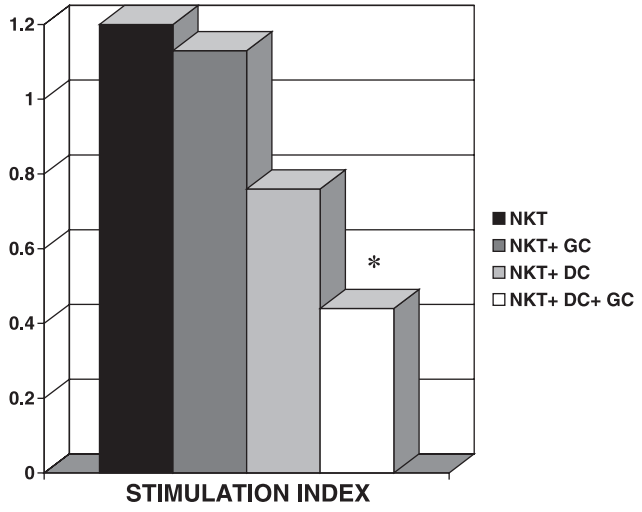

Fig. 1. Effect of glucocerebroside on natural killer T (NKT) cell proliferation in vitro. Incubation with glucocerebroside (GC) led to marked inhibition of NKT cell proliferation in the presence of dendritic cells (DC) but not in their absence. $*P < 0.005$.

Table 2. *Distribution of radiolabeled glucocerebroside 24 and 48 h after oral or intraperitoneal administration (amt)·min^{-1}·100·mg tissue^{-1}*

	Oral		Intraperitoneal	
	24 h	48 h	24 h	48 h
Brain	37	26	33	39
Heart	108	54	64	33
Lungs	79	21	162	63
Liver	765	65	1164	369
Spleen	79	52	627	468
Large bowel	247	83	848	1,017
Small bowel	609	296	1,264	784
Kidney	255	74	407	212

In Vivo Distribution of Radiolabeled GC

Tissue distribution of radiolabeled glucocerebroside was examined 24 and 48 h after intraperitoneal or oral administration (Table 2). After 24 h, increased radioactivity levels were detected in the liver, small intestine, kidney, and colon (765, 609, 255, and 247 cpm, respectively, after oral administration; 1,164, 848, 407, and 1,264 cpm, respectively, after intraperitoneal administration); radioactivity levels were also increased in the same tissues after 48 h (65, 296, 74, 83 cpm for liver, small intestine, kidney, and colon, respectively, after oral administration; 369, 784, 212, and 1,017 cpm, respectively, after intraperitoneal administration). Spleen radioactivity was relatively high (627 and 468 cpm after 24 and 48 h, respectively) after intraperitoneal administration of radioactive GC but not after its oral administration. Radioactivity levels were relatively low in brain, lung, and heart specimens after both oral and intraperitoneal administration of GC.

Effect of GC on ConA Hepatitis

Effect of glucocerebroside on serum ALT and AST levels. Administration of glucocerebroside led to a marked decrease of serum AST and ALT levels in *group A* mice, treated by GC before ConA administration, compared with *group C* mice, that did not receive GC (143 vs. 600 IU, respectively, for serum AST levels; 57 vs. 801 IU, respectively, for serum ALT levels, $P < 0.05$; Fig. 2). Serum AST and ALT levels were decreased to a lesser degree in *group B* animals that were treated with GC after induction of ConA hepatitis (559 and 420 IU, respectively); the difference between serum AST and ALT levels in *group B* compared with *group C* was not statistically significant. Administration of GC to naïve animals (*group D*) did not lead to a significant change in serum AST or ALT levels (175 vs. 144 IU for serum AST and 138 vs. 77 IU for serum ALT in *groups D* and *E*, respectively; *P* values were 0.17 and 0.07, respectively).

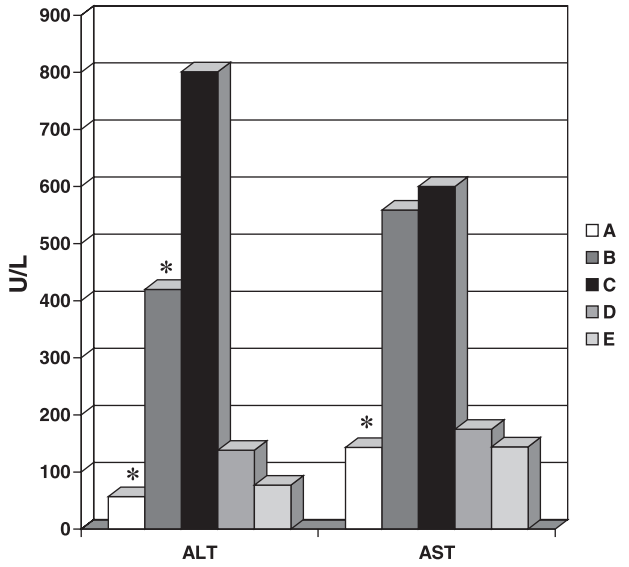

Fig. 2. Effect of glucocerebroside on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Administration of GC led to a significant decrease of serum AST and ALT levels in *group A* mice, treated with GC before Concanavalin A (ConA) administration, compared with *group C* mice that did not receive GC. In the absence of ConA, administration of GC did not induce a significant change in serum AST or ALT levels. $*P < 0.005$.

Effect of glucocerebroside on liver histology. Pathological evidence of liver injury was correlated with serum AST and ALT levels (Fig. 3). Histological liver damage was markedly attenuated in *group A* liver specimens compared with *group B* and *C* liver specimens, in which massive hepatocyte necrosis was present. Livers of *group D* mice, which were treated by GC alone, were identical in appearance to the normal livers of naïve *group E* animals.

Effect of glucocerebroside on serum cytokine levels. Serum IFN-γ was significantly lower in *group A* mice, which were treated by GC 2 h before ConA administration, compared with *group B* and *C* animals (3,725 pg/ml in *group A* vs. 6,220 and 5,620 pg/ml in *groups B* and *C*, respectively, $P < 0.05$; Fig. 4, *A-E*). Serum IFN-γ was negligible in *groups D* and *E* (23 vs. 0 pg/ml, respectively, $P = 0.15$), which did not receive ConA.

Serum IL-2 was significantly higher in GC-treated *group A* mice compared with untreated *group C* animals and did not differ significantly between *groups B* and *C* (602 vs. 273 and 206 pg/ml in groups *A*, *B*, and *C*, respectively; $P < 0.05$, Fig. 4*B*). Serum IL-2 was also significantly elevated in non-ConA-treated *group D* mice that received GC compared with naïve *group E* animals (11 vs. 0.2 pg/ml, $P < 0.05$).

Serum IL-12 was markedly higher in GC-treated *group A* mice compared with untreated *group C* animals and was similar in *groups B* and *C* (22,250, 9,740, and 10,100 pg/ml in *groups A*, *B*, and *C*, respectively; $P < 0.05$, Fig. 4*C*). Serum IL-12 was also significantly elevated in non-ConA-treated *group D* mice, which received GC, compared with naïve *group E* animals (573 vs. 92 pg/ml, $P < 0.05$).

Serum IL-4 was higher in all ConA-treated groups (*A-C*) compared with non-ConA-treated animals (*D* and *E*). There was no significant difference between serum IL-4 levels in *groups A*, *B*, and *C* (31, 34, and 37 pg/ml, respectively; Fig. 4*D*) or in *groups D* and *E* (0 pg/ml in both groups).

There was a trend toward decreased serum IL-10 in GC-treated *group A* mice compared with *groups B* and *C* (8 pg/ml in *group A* vs. 30 and 26 pg/ml in *groups B* and *C*, respectively; Fig. 4*E*) that did not reach statistical significance ($P = 0.07$). Interestingly, in GC-treated naïve mice (*group D*), a trend toward higher IL-10 levels was observed (13 vs. 2.3 pg/ml in *groups D* and *E*, respectively) that did not reach statistical significance ($P = 0.08$).

Effect of glucocerebroside on expression of the transcription factors STAT 1, 4, and 6 and NF-κB. Expression of STAT1, associated with activation of the IFN-γ receptor, was decreased in *group A* (and, to a lesser extent, in *group B*) compared with *group C* and in *group D* compared with *group E* (Fig. 5). Expression of STAT4, classically associated with activation of the IL-12 receptor, was markedly increased in *group A* compared with *groups B* and *C* and slightly increased in *group D* compared with *group E*. Expression of STAT6, associated with activation of the IL-4 receptor, was prominently reduced in

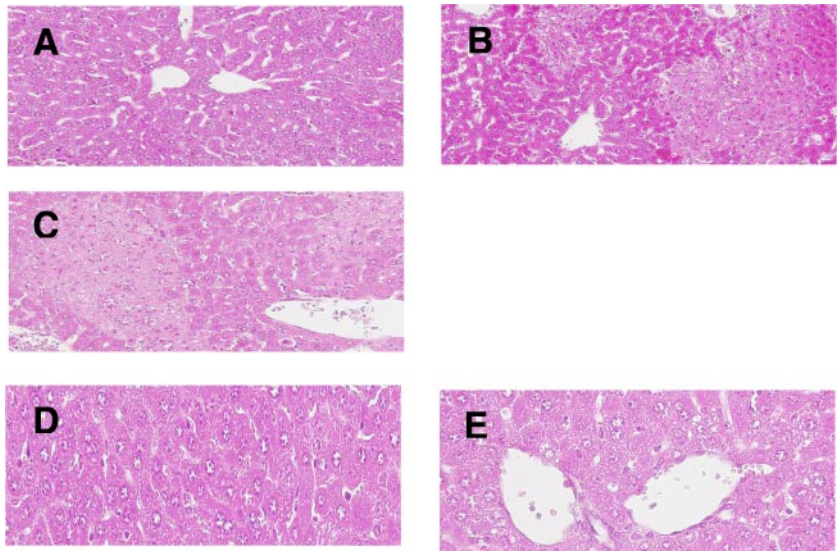

Fig. 3. Effect of GC on liver histology. Histological sections of livers from *group A* mice (*A*) revealed markedly attenuated damage compared with sections of *group C* livers (*C*), in which massive hepatocyte necrosis was present. Livers of *group B* mice (*B*) that were treated with GC after ConA administration were similar in appearance to *group C* livers. Livers of *group D* mice that were treated by GC alone appeared identical to the normal livers of *group E* (naïve) animals (*D* and *E*).

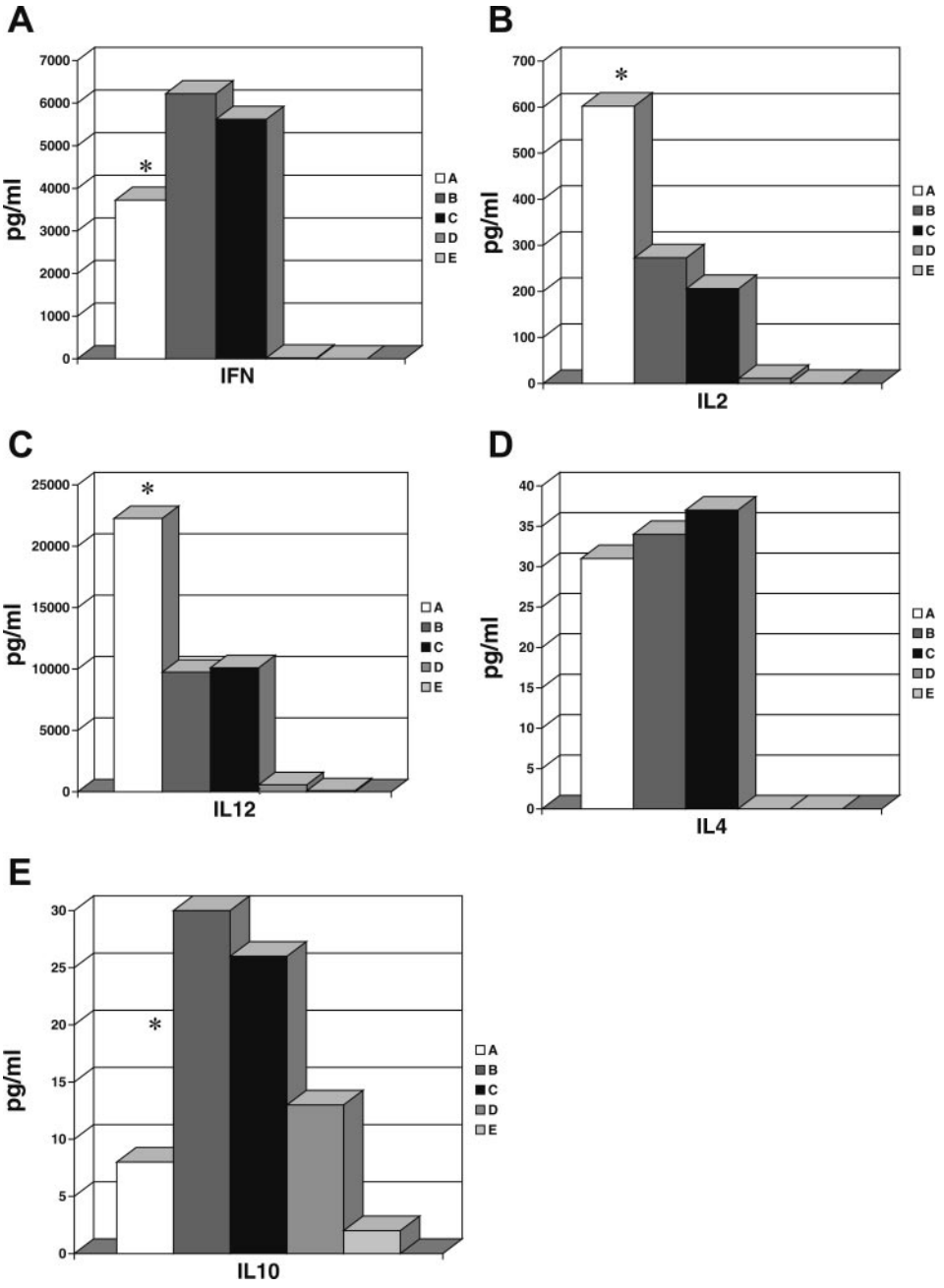

Fig. 4. Effect of GC on serum cytokines. *A*: effect of GC on serum IFN-γ. Serum IFN-γ was significantly lower in GC-treated *group A* mice, compared with untreated *group C* animals. Serum IFN-γ was similar in *groups B* and *C* and negligible in *groups D* and *E* that were not administered ConA. *B*: effect of GC on serum IL-2. Serum IL-2 was increased in GC-treated *group A* mice compared with untreated *group C* controls. *C*: effect of GC on serum IL-12. Serum IL-12 was markedly higher in GC-treated *group A* mice, compared with untreated *group C* animals, and was similar in *groups B* and *C*. Serum IL-12 was also significantly elevated in non-ConA-treated *group D* mice that received GC, compared with naïve *group E* controls. *D*: effect of GC on serum IL-4. Serum IL-4 was higher in all ConA-treated groups (*A-C*) compared with non-ConA-treated groups (*D, E*) and did not differ significantly among groups *A-C*. *E*: effect of GC on serum IL-10. Serum IL-10 decreased in GC-treated *group A* mice compared with untreated *group C* controls. Serum IL-10 was higher in non-ConA-administered, GC-treated *group D* animals compared with naïve *group E* controls. $^*P < 0.05$.

group A (and, to a lesser degree, in *group B*) compared with *groups C-E*. There was no difference in NF-κB expression among the study groups.

Effect of glucocerebroside on splenic and intrahepatic NKT lymphocytes. The effect of glucocerebroside on the number of NKT cells in the liver and spleen was examined. Among ConA-treated groups, administration of GC led to a 24% decrease in the intrahepatic lymphocyte number in *group A*, which was treated by GC 2 h before ConA administration, compared with *group C*, which did not receive GC treatment (2.17% vs. 2.87% NKT lymphocytes in *group A* and *C*, respectively, $P < 0.05$, Fig. 6). An increased NKT cell number was observed in *group B* (3.28%), which was treated with GC after ConA administration. Administration of glucocerebroside to naïve mice (*group D*) led to a slight reduction of the intrahepatic NKT cell number (2.38% vs. 2.51% in *groups D* and *E*, respectively) that was not statistically significant ($P = 0.22$). In the spleen, a small, statistically insignificant increase in the NKT lymphocyte number was observed in GC-treated *group A* mice compared with *group C* animals (5.22% vs. 4.90%, respectively, Fig. 7) and in *group D* mice compared with naïve *group E* controls (4.09% vs. 3.85%, respectively).

DISCUSSION

Although many previous studies have focused on the beneficial effect of NKT lymphocytes and on the therapeutic potential of their activation by α-GalCer in various infectious, neoplastic, and autoimmune clinical settings (30–32), it is now clear that activation of NKT cells can be deleterious to the host.

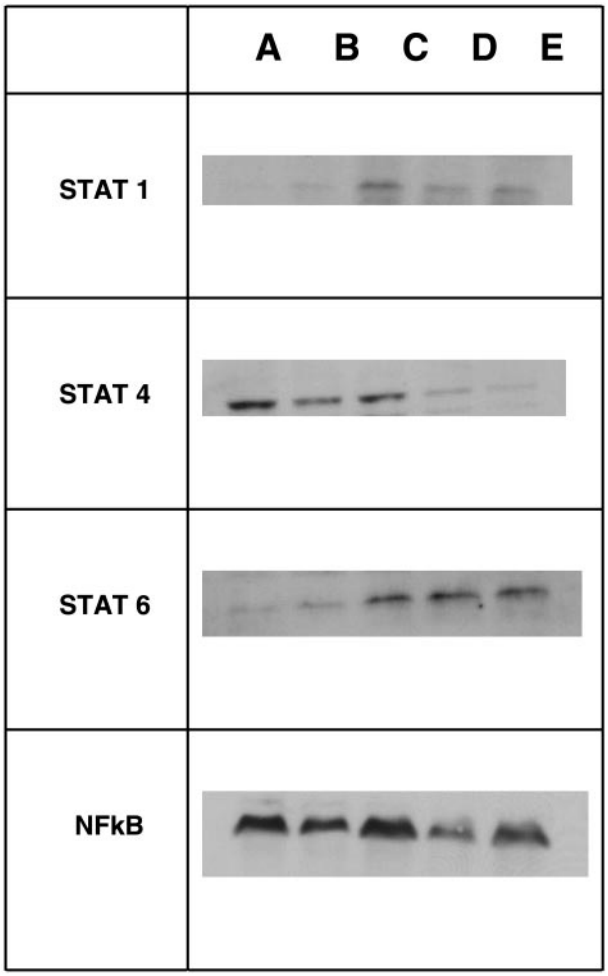

Fig. 5. Effect of GC on expression of the transcription factors STAT 1, 4, and 6 and NF-κB. Expression of STAT1, associated with activation of the IFN-γ receptor, was decreased in *group A* (and, to a lesser extent, in *group B*) compared with *group C* and in *group D* compared with *group E*. Expression of STAT4, classically associated with activation of the IL-12 receptor, was markedly increased in *group A* compared with *groups B* and *C* and slightly increased in *group D* compared with *group E*. Expression of STAT6, associated with activation of the IL-4 receptor, was prominently reduced in *group A* (and, to a lesser degree, in *group B*) compared with *groups C-E*. There was no difference in NF-κB expression among the study groups.

Examples include liver injury induced by ConA, α-GalCer, LPS, and salmonella infection, in which NKT lymphocytes have a key pathogenetic role and suppression of immunity toward certain tumors (33). In such circumstances, inhibition of NKT lymphocytes may prove to be of therapeutic value. Although glucocerebroside may have a role in the proper loading of an activating ligand to CD1d, it was previously suggested that glucocerebroside may prevent activation of NKT cells by α-GalCer (28). The in vitro inhibition of NKT cell proliferation and in vivo amelioration of ConA-induced hepatitis by glucocerebroside observed in this study provide further evidence that glucocerebroside inhibits NKT cell activation.

In vivo, administration of glucocerebroside 2 h before injection of ConA led to marked alleviation of ConA-induced hepatitis, reflected by significantly decreased serum aminotransferase levels and markedly attenuated liver necrosis in

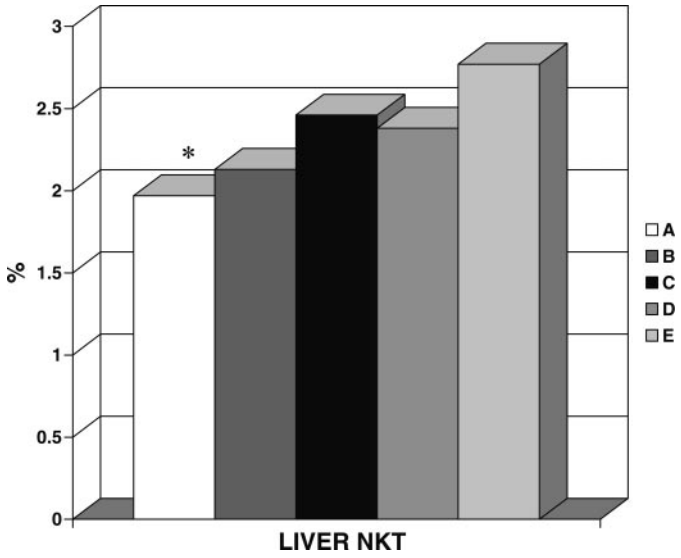

Fig. 6. Effect of GC on intrahepatic NKT lymphocytes. Administration of GC led to a 20% decrease in the intrahepatic NKT lymphocyte number in GC-treated *group A* animals, compared with untreated *group C* controls. A similar effect was observed in non-ConA-administered, GC-treated *group D* mice, compared with naïve *group E* controls ($*P < 0.05$).

GC-treated mice. Although serum transaminases were somewhat reduced in mice treated by glucocerebroside 2 h after ConA administration (*group B*), histological damage and most of the immunological parameters were indistinguishable from those in untreated animals. It is noteworthy that administration of GC to naïve animals did not cause hepatitis; this contrasts with the administration of α-GalCer, which leads to severe hepatitis that shares many features with ConA-induced hepatitis, limiting its therapeutic application (9).

Amelioration of ConA-induced liver damage was associated with decreased serum IFN-γ levels and reduced expression of the transcription factor STAT1, which is associated with acti-

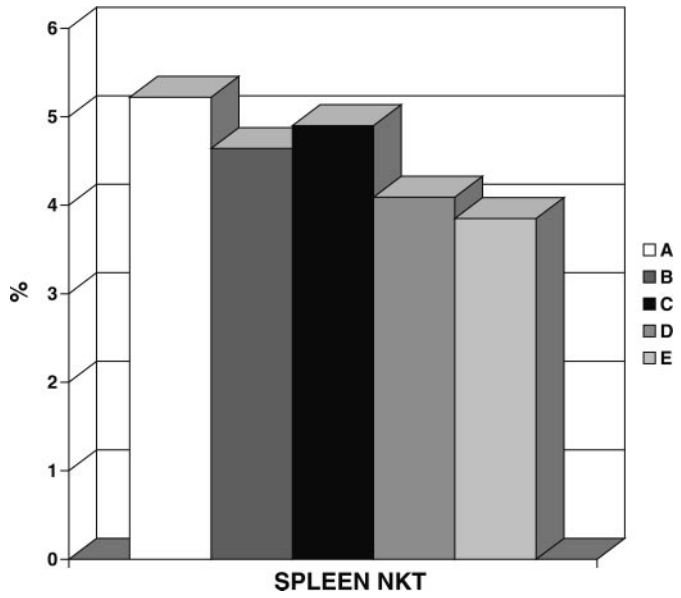

Fig. 7. Effect of GC on peripheral NKT lymphocytes. A slight increase of the peripheral NKT cell number was observed in the spleen of GC-treated animals in the presence or absence of ConA.

vation of the IFN-γ receptor. Together with TNF-α, IFN-γ is considered to have a key role in the pathogenesis of ConA-induced hepatitis (34–37). In addition, the IFN-γ-inducing cytokines IL-12 and IL-18 and macrophage inflammatory protein-2 (MIP-2) contribute to the liver injury in this model (38, 39), whereas IL-6, IL-11, and IL-10 may have a protective role (40, 41). Although it was previously suggested that IL-4 may inhibit acute liver damage and TNF-α production in this model (42), recent studies have shown that anti-IL-4 antibody prevents ConA hepatitis (43) and that Vα14 NKT cells enriched from spleens of IL-$4^{-/-}$ mice and adoptively transferred into Vα14-deficient knockout mice fail to restore susceptibility to ConA, suggesting that IL-4 produced by NKT cells is required for the induction of ConA hepatitis (12). Serum IL-4 did not differ significantly among the ConA-treated groups in the present study; however, STAT6 expression, associated with IL-4 receptor activation, was reduced in *group A* compared with *group C*, implying reduced IL-4 activity in animals in which amelioration of ConA-induced hepatitis occurred. The observed increased levels of serum IL-2 and IL-12 (the latter accompanied by a compatible increased expression of STAT4) and the trend toward decreased IL-10 levels in the clinically responsive group (*group A*) provide further support for the concept that ConA-induced hepatitis involves both Th1 (i.e., IL-2, IFN-γ, and TNF-α)- and Th2 (IL-4, IL-10)-type responses (44).

The beneficial effect of GC in ConA-induced hepatitis was associated with a 20% decrease of the intrahepatic NKT cell number; notably, a similar effect was observed in GC-treated mice that were not exposed to ConA (*group D*). At the same time, a slightly increased peripheral NKT cell number was observed in GC-treated groups. One possible explanation for this finding is redistribution of NKT cells, that is, expulsion of these cells from the liver to the periphery, thus alleviating NKT-mediated liver damage. Other possible explanations for the decreased number of intrahepatic NKT cells in this study may include glucocerebroside-mediated inhibition of NKT cell proliferation, glucocerebroside-mediated apoptosis, and altered subpopulations of NKT lymphocytes. These mechanisms may also underlie the amelioration of ConA-induced hepatitis observed in this study, although a role for non-NKT cell-related mechanisms cannot be excluded.

In vitro, inhibition of NKT cell proliferation was shown to be dependent on the presence of dendritic cells. This supports a requirement for antigen presentation for the inhibitory effect of glucocerebroside, rather than a direct effect of glucocerebroside on NKT cells. One possible explanation for the inhibitory effect of GC observed in the present study is displacement of a yet-uncharacterized natural activating ligand from the CD1d molecule. Binding of glycolipids to CD1d is mediated by anchoring of their lipid tail to the hydrophobic pockets of the CD1d antigen-binding groove (45). Occupation of the CD1d molecule by the ceramide tail of GC, which does not activate NKT lymphocytes, may competitively inhibit binding and presentation of activating ligands in a similar manner to that recently demonstrated for CD1b, another glycolipid-presenting molecule (46). It was recently demonstrated that β-galactosylceramide binds to CD1d without activating NKT cells (47). Interestingly, the mere presence of dendritic cells was also found to decrease NKT lymphocyte proliferation. Because dendritic cells may constitutively express CD1d-bound ligands that could influence NKT cell activation, this effect may have been CD1d mediated. Alternatively, the inhibitory effect of dendritic cells on NKT lymphocyte proliferation may have been unrelated to CD1d-mediated interactions. In a similar manner, the further inhibition of NKT cell proliferation by glucocerebroside could be CD1d related or unrelated.

GC-induced apoptosis may be mediated by glucocerebroside itself or by altered levels of other compounds in the metabolic pathway, such as ceramide, which has a well-characterized proapoptotic effect. Apoptosis of effector cells that mediate ConA-induced liver damage can be expected to ameliorate ConA-induced hepatic injury.

The decreased number of intrahepatic NKT cells and amelioration of ConA-induced hepatitis observed in this study may have resulted from altered proportions of subpopulations of NKT lymphocytes. NKT lymphocytes include subpopulations that are phenotypically and functionally diverse (48). NKT cells identified in this study ($CD3^+DX5^+$) are one population of NKT cells made up mostly of "classic" Vα14 ± NKT lymphocytes; because other surrogate markers for identification of NKT cells were not used, the decreased number of intrahepatic NKT lymphocytes may reflect a relative reduction in the proportion of this subpopulation of NKT cells, rather than a truly lower total NKT lymphocyte number.

After activation of NKT cells, these cells become undetectable in the liver within several hours; repopulation of NKT cells occurs over several days (27). This phenomenon, which may result from apoptosis (27) or from TCR downregulation (49, 50) is thought to underlie the self-limited, transient nature of ConA-induced liver injury, which is characterized by a decline of transaminase levels and commencement of liver regeneration after ~24 h (51). Our findings cannot exclude the possibility that the decreased intrahepatic NKT cell number observed in this study resulted from hastening of activation-induced apoptosis of NKT cells by GC (i.e., more vigorous activation of these cells). Although GC may induce an altered NKT lymphocyte response that is not strictly inhibitory and may differ in various microenvironments and disease states, possibly influenced by the presence of soluble elements and costimulatory molecules, previous data and the complete absence of resultant liver damage in GC-treated animals argue against the possibility that the in vitro inhibition of NKT cell proliferation and in vivo disappearance of intrahepatic NKT cells in this study were activation induced.

GC is normally a constituent of cell membranes; its levels are relatively high in reticuloendothelial tissues (i.e., liver, spleen, and bone marrow) involved in metabolism of senescent blood cells. The relative distribution of radiolabeled GC observed in this study is not in line with straightforward incorporation into cellular membranes or scavenging by reticuloendothelial cells. This notion is supported by the observed accummulation of GC in the small bowel and colon following both oral or intraperitoneal administration. Although hepatic accummulation of GC may have partly resulted from metabolism and excretion of GC by the liver, the observed clinical effect supports an additional role for GC in this organ.

The amounts of GC normally present in the body are vastly greater than those administered in this study. It was previously shown that the average splenic GC content is 0.090 ± 0.047 and 19.9 ± 4.2 mg/g wet tissue in normal subjects and patients with Gaucher's disease, respectively (52). The observed clinical effect in the present study may have resulted from an

altered mode of administration, the presence of "free" rather than bound glucocerebroside, or a different structure of the ceramide tail of naturally occurring murine glucocerebroside, compared with soy-derived glucocerebroside. In contrast to mammalian glucocerebroside, which has a single *trans* double bond at *position 4*, the soy-derived glucocerebroside used in this study has two double bonds at *positions 4* and *8* (65% *trans*, 35% *cis*) (53). Although it could be argued that alleviation of ConA hepatitis by GC may have resulted from direct binding and neutralization of ConA, this possibility appears unlikely in view of the molar ratio of GC to ConA.

In summary, the in vitro and in vivo results of this study suggest that soy-derived glucocerebroside, an easily obtainable, naturally occurring glycolipid, is an immune-modulatory agent that inhibits NKT lymphocyte activity. GC may have a future role in the treatment of autoimmune hepatitis and other immune-mediated disorders, particularly those in which NKT lymphocytes contribute to disease pathogenesis.

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