Amelioration of hepatic fibrosis via betaglucosylceramide-mediated immune modulation is associated with altered CD8 and NKT lymphocyte distribution

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Abstract

Background: While CD8 lymphocytes possess pro-fibrogenic properties and NK (non-T) cells are anti-fibrogenic, the role of NKT lymphocytes in liver fibrosis is still unclear. β-Glucosylceramide (GC), a naturally occurring glycolipid, exerts modulatory effects on these cells. Aim: To explore the role of NKT cells in hepatic fibrosis via GC. Methods: Hepatic fibrosis was induced by biweekly intraperitoneal (IP) carbon tetrachloride (CCl₄) administrations for 7 weeks in 5 groups (A–E) of male C57Bl/6 mice. Mice were treated with daily IP GC injections in groups A and C, or daily oral doses in groups B and D. GC was administered either for the duration of the study period (in groups A and B), or for the last 3 weeks of CCl₄ induction (groups C and D). GC-treated mice were compared with non-treated fibrotic controls (group E) and naive rodents (group F). Liver fibrosis, injury parameters and FACS analysis of lymphocytes were assessed. Results: Marked amelioration (P < 0.0001) of hepatic fibrosis observed in all GC-treated mice without altering reactive oxygen species production. As determined by Sirius red-stained liver tissue sections and measured by Bioquant® morphometry; all CCl₄-administered groups significantly (P < 0.0001) increased the relative fibrosis area compared with naive animals. The increases were 14.4 ± 1.03-fold in group A, 7.9 ± 0.37-fold in group B, 5.2 ± 0.2-fold in group C, 10.3 ± 0.4-fold in group D and 23.8 ± 1.9-fold in group E. Western blot analysis for alpha smooth muscle actin from liver extracts followed a similar pattern, increasing in groups A–E. A significant decrease in liver damage was observed in all GC-treated groups, as noted by a decrease in transaminase serum levels (P < 0.005). The beneficial effect of GC was associated with a significant decrease in the intra-hepatic NKT and CD8 lymphocytes as well as their attenuation of both Th1 and Th2 cytokines. Conclusions: Administration of GC had a significant anti-fibrotic effect following CCl₄ administration. This effect was associated with an altered NKT and CD8 lymphocyte distribution and a cytokine shift. Immune modulation using GC may have a role in the treatment of fibrosis and other immune-mediated disorders.

Introduction

Hepatic fibrosis is the result of chronic liver injury, regardless of etiology, during which hepatic stellate cells (HSCs) proliferate and differentiate into matrix-producing cells (1, 2). Stellate cell activity is affected by an array of cytokines, some of which are pro-fibrotic [e.g. transforming growth factor β1 (3)] while others play an anti-fibrotic role [e.g. IL-10 (4, 5) and interferon-γ (6)]. The role of increased CD8 and decreased CD4 lymphocyte subsets in mediating hepatic fibrosis was previously reported to be attenuated by IL-10 (7). Fibrogenic activity of CD8 lymphocytes was confirmed using the adoptive transfer model of hepatic fibrosis. Furthermore, NK cells were demonstrated to have an anti-fibrotic effect (8). The role of NKT regulatory lymphocytes in fibrogenesis is still unknown. This subset of cells co-expresses cell-surface receptors characteristic of both T lymphocytes (e.g. CD3, α/β TCR) and NK cells (e.g. NK1.1) (9). Activation of NKT lymphocytes can lead to significant liver damage (10, 11). NKT lymphocytes, CD4⁺ T cells and Kupffer cells have a...
contributory role in the mouse model of Con A-induced hepatitis (12–15). NKT cells also have a central role in LPS, α-galactosylceramide (α-GalCer, KRN-7000) and salmonella infection-induced liver injury (16–18), in hepatic injury secondary to deletion of the suppressor of cytokine signaling-1 (SOCS-1) (19) and in hepatic damage in the setting of chronic hepatitis C infection and primary biliary cirrhosis (20, 21). α-GalCer is a potent activator of both mouse and human NKT cells (22). β-glucosylceramide (GC) is a metabolic intermediate in the anabolic and catabolic pathways of complex glycosphingolipids (23). 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) and increased peripheral blood NKT cells (26). CD1d-bound GC does not activate NKT cells directly, and may inhibit activation of NKT cells by CD1d, with secondary inhibition of NKT cell activation (27). On the other hand, glucosylceramide synthase deficiency was shown to lead to defective ligand presentation by CD1d, with secondary inhibition of NKT cell activation (27).

The aim of the present study is to determine the role of NKT cells in an animal model of hepatic fibrosis. In vitro, HSC activation was significantly improved when cultured with splenocytes from GC-treated rather than untreated fibrotic donors. In vivo, administration of GC induces a significant amelioration of hepatic fibrosis associated with a decrease in NKT cells.

Methods

Materials

Carbon tetrachloride (CCl4; Sigma, C-5331) and glatiramer acetate (Copaxone, Teva Ltd) were used in these studies. GC was purchased from Avanti Polar Lipids (Alabaster, AL; cat #131304), dissolved in ethanol and emulsified in PBS.

Animals

Male C57Bl/6 wild-type (WT) mice (8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) 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-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.

Hepatic fibrosis induction

Hepatic fibrosis was induced by intra-peritoneal (IP) CCl4 (diluted to 10% with corn oil) administered as a dose of 5 ml g−1 body weight, for 7 weeks in 8-week-old male C57Bl/6 mice (28).

Experimental groups

Seven groups of mice were studied. Eight animals were included in each group (Table 1). CCl4 was administered for 7 weeks in 5 animal groups (A–E). Mice were treated with daily IP GC injections in groups A and C or daily oral doses in groups B and D. GC was administered either for the duration of the study period as an anti-inflammatory model to prevent fibrosis induction (in groups A and B) or for the last 3 weeks of CCl4 induction to reproduce an anti-fibrotic effect (groups C and D). GC-treated mice were compared with non-treated fibrotic controls (group E) and naive mice (group F).

Table 1. Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>CCl4</th>
<th>GC</th>
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<tr>
<td>A</td>
<td>+</td>
<td>Daily, IP (1.5 μg per mouse) for 7 weeks</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>Daily, PO (15 μg per mouse) for 7 weeks</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>Daily, IP (1.5 μg per mouse) for the last 3 weeks</td>
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<tr>
<td>D</td>
<td>+</td>
<td>Daily, PO (15 μg per mouse) for the last 3 weeks</td>
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<tr>
<td>E</td>
<td>+</td>
<td>—</td>
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<tr>
<td>F</td>
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Liver enzymes

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

Histological assessment of liver injury

The posterior one-third of the liver was fixed in 10% formalin for 24 h and then paraffin embedded in an automated tissue processor. Seven-millimeter slices were cut from the livers of each animal. Sections (15 μm) were stained in 0.1% Sirius Red F3B in saturated picric acid (both from Sigma).

Fibrosis quantitation

Relative fibrosis area (expressed as a percentage of the total liver area) was assessed based on 36 fields from 9 Sirius red-stained liver sections per animal. Each field was acquired at ×10 magnification and then analyzed using a computerized Bioquant® morphometry system. To evaluate the relative fibrosis area, the measured collagen area was divided by the net field area and then multiplied by 100. Subtraction of vascular luminal area from the total field area yielded the final calculation of the net fibrosis area (7).

αSMA immunoblot

Immunoblot analysis of αSMA in liver extracts was performed with modifications as previously described (7). Whole-liver protein extracts were prepared in liver homogenization buffer [50 mmol l−1 Tris–HCl (pH 7.6), 0.25% Triton X-100, 0.15 M NaCl, 10 mM CaCl2, complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. Next, proteins (30 μg per lane) were resolved on a 10% (w/v) SDS-polyacrylamide gel (Novex, Groningen, The Netherlands) under reducing conditions. For immunoblotting, proteins were transferred to a Protran membrane (Schleicher & Schuell, Dassel, Germany). Blots were incubated overnight at 4°C...
in a blocking buffer containing 5% skim milk and then incubated with either anti-SMA (DAKO, cat #M0851) or β-actin (Sigma) mouse monoclonal antibody, diluted 1/2000, for 2 h at room temperature and, subsequently, with peroxidase-conjugated goat anti-mouse IgG (P.A.R.I.S., Compiègne, France), diluted 1/10000, for 1 h at room temperature. Immunoreactivity was revealed by enhanced chemiluminescence using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Les Ulis, France). Bands were scanned (Hewlett-Packard 3400 C) as TIF files (8 bit, 300 dpi) and quantified (Scion Image analysis programme, release beta 4.0.2; Scion Corp, Fredrick, MD, USA). Corresponding β-actin bands were also scanned and the final result was calculated as a ratio of each protein examined to the amount of protein loaded on the electrophoresis gel (29).

CYP2E1 activity
Fifty micrograms of liver was homogenized in 5% trichloroacetic acid at a ratio of 1:10 (w/v) and centrifuged for 5 min at 8000 r.p.m. and 4°C. Catalytic activity of CYP2E1 was determined as the rate of production of p-nitrocatechol from p-nitrophenol (PNP) (30).

Lymphocyte isolation
The spleen was homogenized, and lymphocytes were washed and counted before staining for FACS analysis. Intra-hepatic lymphocytes were isolated by perfusion of the liver with digestion buffer. After perfusion, the liver was homogenized and incubated at 37°C for 30 min. The digested liver cell suspension was centrifuged to remove hepatocytes and cell clumps. The supernatant was then centrifuged to obtain a pellet of cells depleted of hepatocytes and were resuspended in a final volume of 1 ml. Lymphocytes were then isolated from this cell suspension using 24% metrizamide gradient separation (7, 31).

Cytokine measurement
Serum IFNγ, IL-2, IL-12, IL-4 and IL-10 levels were measured in each animal by ‘sandwich’ ELISA, using commercial kits (Genzyme Diagnostics, MA, USA).

FACS analysis
Lymphocytes were adjusted to $2 \times 10^7$ ml$^{-1}$ in staining buffer (in saline containing 1% bovine albumin). Fifty microliters of the cell suspension were incubated with antibody on ice for 30 min, washed with staining buffer and fixed with 2% PFA. Antibodies used for staining were mouse anti-CD4, anti-CD8, anti-CD3 and anti-CD45 antibodies, conjugated by FITC, PE, PerCP and allophycocyanin, respectively. Antibodies were purchased from BD Biosciences, Transduction Laboratories. For determination of the percentage of NKT lymphocytes, anti-CD3 and anti-DX5 antibodies were used (Pharmingen, USA). Intracellular staining of lymphocytes by IFNγ and IL-4 (FITC and PE conjugated, respectively) was performed according to the manufacturer’s protocol (BD Biosciences). Analytical cell sorting was performed on cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson, Oxnard, CA, USA). Only live cells were counted, and background fluorescence from non-antibody-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.

Statistical analysis
Statistical analysis was performed using a two-way analysis of variance. While relative fibrosis area was expressed as mean ± standard error, all other parameters were presented as mean ± SD. Standard error was used in the case of Bioquant® analysis because each group includes 360 readings.

Results
Effect of GC on fibrosis during liver injury—in vivo
Increased Sirius red staining was noted in all CCl$_4$-treated groups (Fig. 1a–e) as compared with naive WT animals (Fig. 1f). Sirius red staining in GC-treated livers (groups A–D)
Different subtypes of lymphocytes are reported to be in an important role in the development of liver fibrosis (7, 8). There is extensive evidence that the immune system plays a crucial role in liver fibrosis. GC altered lymphocyte distribution and cytokine secretion significantly lower in group D as compared with groups A and untreated fibrotic animals (group C). Serum ALT was significantly alleviated in all groups as compared with naive animals and presented as mean ± standard error (Fig. 2). All CCl4-administered groups had a significantly increased relative fibrosis area as compared with naive controls (P < 0.0001). The relative fibrosis area was significantly lower in all GC-treated mice compared with untreated mice (P < 0.0001). Western blot analysis of liver extracts for αSMA as a marker for HSC activation was analyzed in three different membranes. Each membrane contained two different samples from each animal group. Densitometry assessment of αSMA bands were calculated as ratio to its β-actin reading. Therefore, results are presenting mean ratio (±SD) of six animals from each animal group (Fig. 3, upper panel). Results are compatible with relative fibrosis area, showing significant suppression of αSMA expression in GC-treated animals (Fig. 3, lower panel).

CYP2E1 activity (Fig. 4) presented as PNP was significantly lower in the CCl4-treated mice because CCl4 is known to lower CYP2E1 levels via radical inactivation and lipid peroxidation (32). It decreased from 1329.4 ± 36.9 pmol min⁻¹ mg⁻¹ protein in the control mice (group F) to 652.5 ± 134 (P = 0.003), 611.2 ± 263.7 (P = 0.01), 670 ± 55.5 (P = 0.0003), 792.4 ± 39.4 (P = 0.0003), 506.6 ± 122.5 (P = 0.001) in groups A, B, C, D and E, respectively. GC, however, did not affect CYP2E1 activity versus naive and versus non-GC-treated fibrotic mice (P-values were non-significant), indicating that GC does not alter reactive oxygen species (ROS) production by CCl4.

GC ameliorates liver injury
ALT and AST were measured in all groups as serum markers of hepatocyte damage (Fig. 5). Serum ALT and AST levels in the naive WT mice (group F) were 83 ± 41 and 90 ± 50 units, respectively. ALT serum levels increased after fibrosis induction to 223 ± 188 units in group A (P = 0.05), 264 ± 261 units in group B (P = 0.05), 117 ± 96 units in group C (P = 0.2), 74 ± 20 units in group D (P = 0.3) and 7076 ± 1115 units in group E (P < 0.0001). Serum AST also increased after fibrosis induction to 248 ± 271 units in group A (P = 0.1), 458 ± 457 units in group B (P = 0.04), 142 ± 73 units in group C (P = 0.08), 135 ± 52 units in group D (P = 0.08) and 6165 ± 2892 units in group E (P < 0.0001). Serum AST and ALT levels were significantly alleviated in all GC-treated mice (groups A-D, P < 0.0001) as compared with untreated fibrotic animals (group C). Serum ALT was significantly lower in group D as compared with groups A and B (P = 0.03). Serum AST levels were significantly lower in groups C and D as compared with group B (P = 0.04).

GC altered lymphocyte distribution and cytokine secretion
There is extensive evidence that the immune system plays an important role in the development of liver fibrosis (7, 8). Different subtypes of lymphocytes are reported to be involved in liver fibrosis as well as in the clearance of necrotic cells during inflammation (33, 34). GC has been shown to modulate NKT cells function (22). In the present study, the proportion of NKT lymphocytes significantly increased from
0.95 ± 0.3% of CD45\(^+\) cells in naives to 5.8 ± 1.6% following fibrosis induction (Fig. 6a, upper panel, \(P < 0.0001\)). GC treatment from group C was associated with a significant decrease of intra-hepatic NKT to 0.71 ± 0.28\% (\(P < 0.0001\) versus non-GC fibrotic group and \(P = 0.08\) versus naives). The total number of NKT cells followed same pattern seen in its distribution (Fig. 6a, lower panel, \(P < 0.0001\)).

The proportion of CD4 lymphocytes (presented as percentage of CD45\(^+\) cells) was significantly reduced in fibrotic groups (C and E) compared with naive mice (group F) (\(P = 0.01\)); CD4 T cells were 14.5 ± 3.5\% of CD45\(^+\) cells in naive mice, 10.3 ± 2.3 in CCl\(_4\) mice and 10.9 ± 2.1 in CCl\(_4\) + GC mice (Fig. 6b, upper panel). The number of liver CD4 cells, however, was significantly increased in both fibrotic groups (Fig. 6b, lower panel). They increased from 0.29 ± 0.12 \(\times\) 10\(^6\) cells in naives to 0.62 ± 0.26 and 0.56 ± 0.16 million cells in non-treated and GC-treated groups, respectively (\(P\)-values were 0.003 and 0.001, respectively). Both fibrotic CD4 counts were similar (\(P = \text{not significant}\)).

IFN\(_c\)-secreting CD4 cells (IFN\(_c\)^+CD4\(^+\)) were 4.2 ± 2.5\% of total CD4 cells in naive mice. IFN\(_c\)^+CD4\(^+\) cells significantly increased to 21.2 ± 5.7\% following fibrosis induction (\(P < 0.0001\)). GC treatment was associated with a significant decrease in IFN\(_c\)^+CD4\(^+\) cells to 6.2 ± 1.7\% (\(P < 0.0001\) versus group E and \(P = 0.06\) versus naive mice). IL-4\(^+\)CD4\(^+\) cells were 5.7 ± 3.8\% of total CD4 cells in naive mice, increased to 14.6 ± 7.2\% in the fibrotic group (\(P = 0.01\)) and significantly decreased to 3.9 ± 1.8\% in GC-treated animals (\(P = 0.0008\) versus group E and \(P = 0.1\) versus naive mice). In spite of the increased hepatic CD4 number in both fibrotic groups, the numbers of both IFN\(_c\)^+CD4\(^+\) and IL-4\(^+\)CD4\(^+\) cells significantly decreased following GC treatment mimicking the pattern seen in their proportions (Fig. 6b, lower panel).

The average percentage (and total number) of CD8 cells was 9.4 ± 3.9\% of CD45\(^+\) cells (with total of 0.29 ± 0.1 million cells) in naive animals, and this was significantly (\(P = 0.004\)) increased to 15.4 ± 2\% (0.85 ± 0.1 million cells, \(P = 0.004\)) in CCl\(_4\)-treated mice (Fig. 6c). GC treatment from group C decreased the CD8 proportion to 12.8 ± 2.2\% (\(P = 0.04\)) and total number to 0.57 ± 0.08 millions (\(P = 0.03\), as compared with CCl\(_4\) group. Following fibrosis induction, the proportion of IFN\(_\gamma\)^+CD8\(^+\) T cells increased...
from 5.1 ± 2.9% of total CD8+ lymphocytes to 19 ± 3.1% in fibrotic mice (P < 0.0001; GC treatment was associated with a significant decrease to 6.8 ± 2.7% (P < 0.0001), similar to that noted in naive animals (P = 0.2). IL-4+CD8+ cells increased by 5.9 ± 3.8% in naive to 20.5 ± 15.7% in CCl4-treated animals (P = 0.04). GC administration led to a decrease to 4.9 ± 1.7% (P = 0.01). Both IFNγ+CD8+ and IL-4+CD8+ cells significantly decreased following GC treatment mimicking the pattern seen in their proportions (Fig. 6c, lower panel).

All other GC-treated groups (A, B and D) showed similar NKT, CD4 and CD8 alterations seen in group C (data not shown). A non-significant effect was noted on lymphocytes harvested from spleens in the different groups (data not shown).

**Serum cytokine levels were reduced in GC-treated animals**

Induction of fibrosis was associated with a significant increase in IFNγ serum levels from 0.4 ± 1 pg ml⁻¹ in naive animals (group F) to 26.8 ± 5.7 pg ml⁻¹ in group A, P < 0.0001, (Fig. 7), 70 ± 9.9 pg ml⁻¹ in group B, P < 0.0001, 34 ± 20 pg ml⁻¹ in group C, P < 0.0001, 14.5 ± 6.4 pg ml⁻¹ in group D, P < 0.0001, and 27.3 ± 20.9 pg ml⁻¹ in group E, P = 0.002. Comparing to the non-treated mice, GC treatment was only associated with significant alterations in IFNγ levels in group B (P = 0.03). This IFNγ value in group B, when compared with other GC-treated groups, was increased significantly (P = 0.001 compared with group A, 0.05 compared with B and 0.01 compared with D). Serum IFNγ concentrations were significantly lower in group D when compared with mice in groups A and B (P = 0.04 and 0.01, respectively).

As is seen in IFNγ, induction of fibrosis also leads to a significant increase in the IL-4 serum concentration from 10.1 ± 3.1 pg ml⁻¹ in naive animals (group F) to 27.3 ± 7.5 pg ml⁻¹ in group A, P = 0.003, 27.5 ± 3.5 pg ml⁻¹ in group B, P = 0.0001, 21.3 ± 10.9 pg ml⁻¹ in group C, P = 0.01, 18 ± 8.6 pg ml⁻¹ in group D, P = 0.02 and 27.5 ± 24.7 pg ml⁻¹ in group E, P = 0.03, (Fig. 7). No significant differences in IL-4 levels were detected in any of the five fibrogenic groups (A–E).

Serum IL-10 levels were 2.9 ± 5.5 pg ml⁻¹ in naive animals (group F), 11.5 ± 14.8 pg ml⁻¹ in group A, 18.5 ± 26.2 pg ml⁻¹ in group B, 5.4 ± 6.9 pg ml⁻¹ in group C, 12 ± 21 pg ml⁻¹ in group D and 13 ± 25 pg ml⁻¹ in group E. Compared with naive mice, only mice in group B manifested a significant increase in IL-10 serum levels (P = 0.05, Fig. 7).

In summary, GC treatment did not alter cytokine serum levels except the case of IFNγ in group B, suggesting that the intra-hepatic decrease in cytokine excretion by lymphocytes are affecting the liver locally and are not affecting the systemic circulation.

**Discussion**

Recent studies have suggested that the immune system plays a role in fibrogenesis. We have previously explored the impact of IL-10 on CD8 and CD4 lymphocyte subsets in experimental liver injury (7). In a transgenic mouse model with hepatocyte secretion of rat IL-10, the anti-fibrotic effect of IL-10 was established, which was associated with re-duced CD8 lymphocytes. Adoptive transfer of CD8 lymphocytes from mice with liver injury could initiate fibrosis in naive mice. In contrast to CD8 lymphocytes, NK cells exerted an anti-fibrotic effect (8). Collectively, these findings broaden our understanding of how immune mediation can impact on fibrosis, and point to manipulation of CD8, CD4 and NK subsets as a potential means for therapeutically modulating fibrosis.

Several studies have focused on the beneficial effect of NKT lymphocytes and on the therapeutic potential of their activation by αGalCer in various infectious, neoplastic and autoimmune clinical settings (35–38). However, the role of NKT cells in hepatic fibrosis is still unclear.

The results of the present study suggest that GC can lead to significant attenuation of hepatic fibrosis in a CCl4 animal model, and its beneficial effect is associated with altered intra-hepatic NKT regulatory lymphocyte distribution. The anti-fibrotic activity was demonstrated by Sirius red staining, Bioquant densometry and αSMA western blotting from liver extracts (Fig. 1–3). Anti-fibrotic effects in groups A and B were preventive, while the effect of GC in groups C and D reflects its ability to reverse an ongoing fibrosis. CCl4 is known to lower CYP2E1 levels via radical inactivation and lipid peroxidation (32, 39). GC, however, did not affect CYP2E1 activity; indicating that GA do not alter ROS production by CCl4. The anti-fibrotic activity was associated with an anti-inflammatory effect. Serum AST and ALT levels were decreased in all GC-treated mice. These beneficial effects were associated with an altered total number of lymphocyte subset and their distribution, manifested by a significant decrease in the pro-fibrotic CD8 subset, and a decrease in both IFNγ- and IL-4-secreting CD4 and CD8 lymphocytes in the liver (Fig. 6). The fact that all other GC-treated groups (A, B and D) showed a similar NKT, CD4 and CD8 alterations seen in group C (data not shown) can explain why four different treatment protocols (Table 1) were similarly effective in ameliorating the fibrosis. Lymphocytes expressing high levels of anti-fibrosis cytokines such as...
IL-10 or IFNγ were previously shown to exert an anti-fibrotic effect (6, 7), while the lymphocytes expressing high levels of pro-fibrosis cytokines such as IL-4 have a pro-fibrotic role (40–42). Fibrosis induction in the CCl₄ animal model was associated with an increase of intra-hepatic NKT cells. Amelioration of fibrosis by GC was accompanied by NKT reduction, indicating that NKT regulatory cells are pro-fibrogenic in this setting. Administration of α-GalCer was reported to lead to rapid production of both IFNγ and IL-4 by NKT cells, with secondary activation of innate and adaptive immune responses (43, 44). GC was shown to have an immunomodulatory effect on NKT cells. In an in vitro study, exposure of NKT cells to GC in the presence of dendritic cells inhibited NKT cell proliferation. GC was recently shown to enhance NKT plasticity in immunologically opposed models. In Th-1 mediated pro-inflammatory conditions such as immune-mediated colitis and non-alcoholic steatohepatitis models, administration of GC was associated with a Th₁ to Th₂ shift and amelioration of target organ damage (45). In contrast, in the hepatocellular carcinoma model administration of GC induced a Th-1 immune shift and tumor suppression (46). Furthermore in Con A immune-mediated hepatitis, a model in which NKT mediated the liver damage, administration of GC ameliorated the disease in association with a decrease in the intra-hepatic level of NKT (47).

In the present study, the beneficial effect of GC was associated with a decreased intra-hepatic NKT cell number. One possible explanation for this finding is re-distribution of NKT cells, that is—expulsion of these cells from the liver to the periphery, thus alleviating NKT-mediated liver fibrosis. Other possible explanations for the decreased number of intra-hepatic NKT cells in this study may include GC-mediated inhibition of NKT cell proliferation or GC-mediated apoptosis and altered sub-populations of NKT lymphocytes. NKT lymphocytes include sub-populations that are phenotypically and functionally diverse. The CD3⁺DX5⁺NKT cells identified in this study are one population of NKT cells, made up mostly of ‘classical’ Vα14⁺ NKT lymphocytes. As other surrogate markers for identification of NKT cells were not used, the decreased number of intra-hepatic NKT lymphocytes may reflect a relative reduction in the proportion of this sub-population of NKT cells, rather than a decrease in the total number of NKT lymphocytes.

The NKT-associated GC-mediated anti-fibrotic effect observed in the present study is consistent with several clinical observations. Gaucher's disease is a lysosomal storage disorder caused by deficiency of the enzyme glucocerebrosidase and characterized by the presence of pathological macrophages laden with glucosylceramide (48–50). Hepatomegaly is frequently observed in type 1 Gaucher's disease. In a series of 53 patients (51), 41 had hepatomegaly, which was classified as mild in 27 patients, moderate in nine and massive in five. but only 10 of these patients had abnormal serum tests of liver function and only two suffered complications of portal hypertension (52). In a comprehensive examination of the hepatic disease that occurs in non-neuronopathic Gaucher’s disease, James et al. (53) conducted a systematic survey of the liver abnormalities in a cohort of 21 patients with type 1 Gaucher’s disease. All but one of these patients had enlarged livers (2 mild, 10 moderate and 8 massive), and liver biopsies showed that all patients had Gaucher cells present in the liver parenchyma. Hepatic fibrosis was not prominent in any of these patients, and the portal hypertension was referred to the infiltrative pattern of the disease. Therefore, for most patients, infiltration of the liver with Gaucher cells has no significant clinical consequences. There have, however, been sporadic case reports of portal hypertension in patients with Gaucher’s disease (54–64). These patients appear to fall into two groups: those with intact spleens (55–58, 64) in whom the portal hypertension can be alleviated by splenectomy (56, 58, 60–63) where intra-hepatic obstruction to portal blood flow has been associated with massive infiltration of the liver by Gaucher cells, with associated centrilobular hepatic fibrosis but not overt cirrhosis (56, 59, 61, 63, 65).

A significant increase in IFNγ and IL-4 serum levels was noted following fibrosis induction. GC treatment did not alter this pattern except in group B (oral administration of GC for duration of study period). Mice in group B manifested a significant increase in IL-10 serum levels. Cytokines act locally and possess very short half-lives (66); therefore, most cyto- kinase activity occurs in cells that are in close proximity to the sites of cytokine formation. Cytokine profiles in liver compartments may vary depending on the stage of hepatic disease of a particular individual, sometimes with high amounts of antigen biasing toward a larger Th1 response (67). Alternatively, these data suggest that GC may exert an effect that is independent of serum cytokine levels.

In summary, soy-derived GC is an easily obtainable, naturally occurring glycolipid, which attenuates hepatic fibrosis. The beneficial effect was associated with a significant decrease in intra-hepatic NKT and CD8 lymphocyte subsets. These results further support a role for these cells in fibrogenesis. GC may have a future role in the treatment of hepatic fibrosis and other immune-mediated disorders, particularly those in which NKT lymphocytes contribute to disease pathogenesis.

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Abbreviations
αSMA α-smooth muscle actin
ALT alanine aminotransferase
AST aspartate aminotransferase
CCCl₄ carbon tetrachloride
α-GalCer α-galactosylceramide
GC glucosylceramide
HSC hepatic stellate cells
IP intra-peritoneal
PNP p-nitrophenol
ROS reactive oxygen species
WT wild type

References
Treatment of hepatic fibrosis by glucosylceramide


