Beneficial effect of glatiramer acetate (Copaxone) on immune modulation of experimental hepatic fibrosis


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Horani A, Muhanna N, Pappo O, Melhem A, Alvarez CE, Doron S, Wehbi W, Dimitrios K, Friedman SL, Safadi R. Beneficial effect of glatiramer acetate (Copaxone) on immune modulation of experimental hepatic fibrosis. Am J Physiol Gastrointest Liver Physiol 292: G628–G638, 2007. First published October 12, 2006; doi:10.1152/ajpgi.00137.2006.—While CD8 subsets activate hepatic stellate cells and reduced serum IL-4 levels and increased NK cells, the antifibrotic effect of IL-10 was established, which was associated with fewer CD8 lymphocytes. Moreover, adoptive transfer of CD8 lymphocytes from mice with liver injury was found to initiate fibrosis in naive mice. In contrast to CD8 lymphocytes, NK cells exert an antifibrotic effect (40, 46). Collectively, these findings broaden our understanding of how mediation by various immune factors can affect fibrosis and point to the manipulation of the CD4, CD8, and NK subsets as a potential means of therapeutically modulating fibrosis.

Glatiramer acetate (GA; also known as Copaxone or copolymer 1) is a synthetic copolymer drug, composed of a random mixture of four amino acids, used in the treatment of multiple sclerosis (MS) (7, 70). GA is a well-tolerated drug that is considered among the safest of the currently approved therapeutic agents for treating MS. Importantly, long-term treatment with GA does not lead to hematologic or liver enzyme abnormalities (27). However, the exact underlying mechanism by which GA exerts its effect in MS still remains elusive. Nevertheless, there is a growing body of evidence that GA reduces the relapse frequency of MS through its effect on dendritic cells (46, 66). In experimental autoimmune encephalitis, the immunologic cross-reaction between the myelin basic protein and GA is thought to be the basis for the suppressive activity of GA, through the induction of antigen-specific suppressor antibodies to IL-10 receptors induce an increase in fibrosis (68) and is supported by the analysis of the effect of fibrogenic drugs on IL-10-knockout mice (62) and by attenuation of experimental fibrosis following electroporative IL-10 gene therapy or transgenic IL-10 (10, 54).

Different inflammatory cells including natural killer (NK) cells, Kupffer cells, and lymphocytes each play a distinct role in the interplay of pro- and antifibrotic stimuli in normal liver and after injury. Lymphocytes express high levels of antifibrogenic cytokines such as interferon-γ and thus have an antifibrotic profile (58, 68), whereas lymphocytes that express high levels of fibrogenic cytokines such as IL-4 have a profibrotic role (58, 69). Therefore, subtle alterations in the intrahepatic cytokine ratio can titrate the intrahepatic milieu toward or away from fibrosis (22, 23).

We previously explored (47, 54) the impact of IL-10 on CD8 and CD4 lymphocyte subsets in experimental liver injury. To this end, we generated a transgenic mouse with hepatocyte secretion of rat IL-10, to assess the impact of sustained local expression of the cytokine on hepatic fibrogenesis. An antifibrotic effect of IL-10 was established, which was associated with fewer CD8 lymphocytes. Moreover, adoptive transfer of CD8 lymphocytes from mice with liver injury was found to initiate fibrosis in naive mice. In contrast to CD8 lymphocytes, NK cells exert an antifibrotic effect (40, 46). Collectively, these findings broaden our understanding of how mediation by various immune factors can affect fibrosis and point to the manipulation of the CD4, CD8, and NK subsets as a potential means of therapeutically modulating fibrosis.

Hepatic fibrosis is the result of chronic liver injury, regardless of etiology, during which hepatic stellate cells (HSC) proliferate and differentiate into matrix-producing myofibroblastic cells (17, 18). The activity of stellate cells is influenced by an array of cytokines, some of which are profibrotic, e.g., transforming growth factor (TGF) (21), whereas others play an antifibrotic role, e.g., interleukin (IL)-10 (62, 68) and interferon (58, 64). The antifibrotic effect of IL-10 is further underscored by studies of cultured stellate cells in which...
The animals received care according to the National Institutes of Health guidelines. The animal procedures were approved by the local Institutional Animal Care and Use Committee.

**MATERIALS AND METHODS**

**Materials.** CCl4 from Sigma (C-5331) and GA (Copaxone) from Teva were used in these studies.

**Animals.** Eight-week-old male C57BL/6 wild-type mice were used. The animals received care according to the National Institutes of Health guidelines. The animal procedures were approved by the local Institutional Animal Care and Use Committee.

**Hepatic fibrosis induction.** Hepatic fibrosis was induced by intraperitoneal CCl4 (diluted to 10% with corn oil) administered as a dose of 5 μl/g body wt twice a week for 6 wk in 8-wk-old male wild-type C57BL/6 mice (26, 54). During the last 2 wk, the animals were also treated with an intraperitoneal injection of either 200 μg of GA or medium per day. Both groups were compared with naive wild-type mice. Eight animals were included in each group. After ketamine-xylazine anesthesia, the animals were euthanized 3 days after the final treatment. Blood samples were obtained and frozen at −20°C until being assayed for cytokine levels. Livers were divided for mRNA analysis and stored at −80°C for staining, and the rest were used for isolating lymphocytes. Splenocytes and intrahepatic lymphocytes were isolated for fluorescence-activated cell sorting (FACS) analysis from all animal groups as detailed below.

**Biochemistry.** Sera from individual mice were obtained. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by an automatic analyzer.

**Liver histology.** The posterior one-third of the liver was fixed in 10% formalin for 24 h and then paraffin-embedded with an automated tissue processor. Seven-millimeter sections were cut from each liver specimen. Hematoxylin and eosin staining was performed for each animal. The intrahepatic necroinflammatory score was evaluated blindly by a histopathologist using the standard histology activity index as described by Knodell et al. (33). Liver sections (15 μm) were also stained in 0.1% Sirius red F3B in saturated picric acid (both from Sigma) (54) for computerized Bioquant morphometry system analysis. For the in situ detection of HSC apoptosis, liver sections were double stained with annexin V and α-smooth muscle actin (SMA) according to manufacturer's instructions. Liver sections were analyzed by confocal microscopy (40).

**Hepatic fibrosis quantification.** Hepatic fibrosis was assessed with a computerized Bioquant morphometry system (24, 25, 54, 67). Immunoblotting (using Western blotting) and semiquantitative PCR analysis of α-SMA mRNA assessed liver extracts, as previously described (54) and as detailed below.

**Relative fibrosis area.** Relative fibrosis area was assessed by analyzing 56 Sirius red-stained liver sections per animal. Each field was acquired at ×10 magnification and then analyzed with a computerized Bioquant morphometry system. To evaluate the relative fibrosis area, we divided the measured collagen area by the net field area and then multiplied it by 100. Subtraction of the vascular luminal area from the total field area yielded the final calculation of the net fibrosis area.

Western immunoblotting. Whole liver protein extracts were prepared in liver homogenization buffer (50 mM Tris·HCl (pH 7.6), 0.25% Triton X-100, 0.15 M NaCl, 10 mM CaCl2, and a complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. Next, proteins (30 μg/lane) were resolved on a 10% (wt/vol) 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 an anti-SMA mouse monoclonal antibody (Dako, catalog no. M0851), diluted 1/2,000 and maintained for 2 h at room temperature and subsequently diluted 1/10,000 with peroxidase-conjugated goat anti-mouse IgG (PARIS, Compiegne, France) and maintained for 1 h at room temperature. Immunoreactivity was revealed by enhanced chemiluminescence with an ECL kit (Amersham Pharmacia Biotech, Les Ulis, France) (52). For tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), incubation with anti-TRAIL-R2 antibodies (dilution 1:1,000; AB1687 or AB16942; Chemicon, Temecula, CA) was used (5).

**Tissue RNA extraction and α-SMA mRNA detection.** Total cellular RNA was extracted from frozen liver tissues with TRIzol reagent (GIBCO-BRL, Life Technologies) and DNA digestion. RNA was then used as a template for reverse transcription into single-stranded cDNA with the Reverse Transcription System (Promega, Madison, WI). mRNAs for α-SMA and β-actin were detected by quantitative real-time PCR with the following primers: β-actin (as a housekeeping gene): forward 5′-GAT GAG ATT GGC ATG GCT TT-3′, reverse 5′-AGA GAA GTG GGG TGG CTT TT-3′; α-SMA: forward 5′-TCC TTC CTG GAG AAG AGC TAC-3′, reverse 5′-TAT AGG TGG TTG CGT GGA TGC-3′ (54); TRAIL: forward 5′-CCC TGC TTG CAG GTT AAG AG-3′, reverse 5′-GGC CTA AGG CCT TCT CAT CC-3′ (46).

**Real-time PCR analysis of fibrogenic markers.** Synthesis of cDNA was performed with 2 μg of total RNA per sample with random primers and reagents contained in the Reverse Transcription System kit, according to the manufacturer’s protocol (Promega). The reverse transcriptase product was diluted 20× in nuclease-free H2O, and 5 μl of each sample was loaded into 96-well plates for real-time PCR in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). β-Actin served as internal control, and H2O served as a negative control. Amplification reactions included oligonucleotide primers for each target gene and for β-actin, as well as platinum Taq polymerase and SYBR Green DNA-binding dye. Fluorescence signals were analyzed during each of 40 cycles (denaturation 15 s at 95°C, annealing 15 s at 56°C, and extension 40 s at 72°C). Denaturation curves of target genes and β-actin, performed at the end of the PCR, and detection of the PCR products by agarose gel electrophoresis confirmed the homogeneity of the DNA products. Relative quantification was calculated with the comparative threshold cycle (Ct) method (as described in User Bulletin no. 2, ABI PRISM 7700 Sequence Detection System). Ct indicates the fractional cycle number at which the amount of amplified target genes reach a fixed threshold within the linear phase of gene amplification and is inversely related to the abundance of mRNA transcripts in the initial sample. Mean Ct of duplicate measurements would be used to calculate ΔCt as the difference in Ct for target and reference. ΔCt for each sample was compared with the corresponding control Ct, and the result was expressed as ΔΔCt. Relative quantity of product was expressed as fold induction or repression of the target gene compared with the control primers, according to the formula 2−ΔΔCt.

**Cytochrome P-450 2E1 activity.** Fifty micrograms of liver was homogenized in 5% trichloroacetic acid at a ratio of 1:10 (wt/vol) and centrifuged for 5 min at 8,000 rpm and 4°C. Catalytic activity of cytochrome P-450 2E1 (CYP2E1) was determined as the rate of production of p-nitroacetaldehyde from p-nitrophenol (49).
HSC isolation. HSC were isolated from all groups by sequential pronase/collagenase digestion followed by Nycozen-d density gradient centrifugation as described previously with minor modifications in rats (19, 20). After anesthesia and abdominal exploration, liver perfusion via the portal vein with 20 ml MEM (GIBCO BRL) obtained hepatic washout. Perfusion was then followed by 10 ml of DMEM-F-12 (GIBCO BRL) containing 0.5 mg Pronase (Roche Diagnostics, catalog no. 1459643) per gram of body weight of the mouse, followed by 10 ml of DMEM-F-12 containing 7 mg of Liberase enzyme 3 (Roche Diagnostics, catalog no. 1814184). The digested liver was washed ex vivo and was incubated at 37°C for 25 min in 50 ml of DMEM-F-12 solution containing 0.05% Pronase and 20 μg/ml of DNase I (Roche Diagnostics, catalog no. 1284932). The resulting suspension was filtered through a 150-μm steel mesh and centrifuged on an 8.2% Nycozen-d (Sigma catalog no. D-2158) cushion at 1400 g for 15 min, which produced a stellate cell-enriched fraction in the upper whitish layer. Samples were stored at 4°C until the FACS analysis was performed.

Cell isolation, staining, and flow cytometric analysis. Spleens were harvested at the time of euthanasia and fractionated through a 70-μm nylon cell strainer. After red blood cell lyses, the splenocytes were washed, suspended in RPMI 1640 medium, and stored at 4°C until FACS analysis. Intrahepatic 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. Next, 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 to a final volume of 1 ml. Lymphocytes were then isolated from this cell suspension by 24% metrizamide gradient separation (39, 54). Human peripheral blood was collected in heparin from patients and healthy control subjects after Institutional Review Board approval. Mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (Pharmacia) according to Boyum (9). After three washes in saline, the cells were resuspended in RPMI 1640 medium supplemented with 50% heat-inactivated fetal calf serum (GIBCO) and 20% dimethyl sulfoxide; cells were stored at −80°C.

FACS analysis. Briefly, lymphocytes were adjusted to 2 × 10^7/ml in staining buffer (in saline containing 1% bovine albumin), incubated for 30 min at 4°C with antibodies conjugated to fluoroscein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC), washed three times, and then resuspended in fixative solution with 2% paraformaldehyde for analysis. Lymphocytes were stained with monoclonal anti-mouse CD4 and CD8 antibodies conjugated by PE and FITC, respectively (BD Biosciences, San Jose, CA). For staining NK cells, we used APC-conjugated rat anti-mouse CD49b/Pan-NK cells possessing monoclonal antibody that identifies the majority of the NK cells. NK cells were defined as CD49b+CD3− cells, and therefore PE-conjugated rat anti-mouse CD3 staining was used. Staining with peridinin chlorophyll-α protein (Per-Cy5)-conjugated rat anti-mouse CD45 (BD Biosciences) was used to identify lymphocytes. T regulatory (Treg) cells were defined as CD4+CD25+FoxP3+ with PE-conjugated rat anti-mouse CD25 and APC-conjugated rat anti-mouse FoxP3. For apoptosis measurements of freshly isolated HSC, propidium iodide (PI) staining of fragmented DNA (42) and phosphatidylserine staining by annexin-V conjugated to FITC (65) (R&D Systems, Minneapolis, MN) were used according to the manufacturer’s instructions. HSC apoptosis was therefore defined as annexin-V+ but PI−. FACS data were acquired with a FACS Calibur flow cytometer (BD Biosciences) set to capture all events.

In vitro human study. Peripheral blood lymphocytes from hepatitis C virus (HCV)-cirrhotic patients versus normal controls were cocultured 48 h with human HSC (LX2 cells; Ref. 61). Before coculture with LX2 cells, HCV-derived lymphocytes were preincubated with 1 μg/ml GA or medium and washed. Triplicates from eight patients were cultured under each condition. After 48 h of incubation, HSC were washed and lymphocyte-free HSC were harvested with a scraper. Protein lysate of the obtained HSC was assessed by Western blotting of α-SMA and β-actin.

Cytokine measurements. To measure IL-4, interferon (INF)-γ, and IL-10 levels, OptEIA ELISA kits (Pharmingen, San Diego, CA) were used according to the manufacturer’s protocol. A standard curve was generated with recombinant cytokines, and concentrations of samples were determined by using a polynomial curve fit analysis.

Statistical analysis. Lymphocyte subsets, serum aminotransferase levels, and the fibrosis area of each of the groups were analyzed for significance with ANOVA and Student’s t-test. Correlations were analyzed with Pearson’s pairwise correlation. Data were analyzed with JMP statistical software version 5.0 (SAS Institute, Cary, NC). The results are presented as means (SD); SE was used in the case of Bioquant analysis because each group includes 360 readings.

RESULTS

GA decreases CCl4-induced hepatic fibrosis. All animal groups survived 6 wk of CCl4 treatment and were analyzed for the extent of fibrosis by morphometry. GA treatment in the final 2 wk of CCl4 mimics a real antifibrotic effect since fibrosis is advanced and well established at the end of the week (54). GA treatment significantly attenuated hepatic fibrosis, since fibrotic septa were more established in CCl4-treated mice that did not receive GA compared with those treated with GA based on Bioquant morphometry (Fig. 1A). More specifically, hepatic fibrosis decreased from 5.28±0.32% (mean±SE) and 2.85±0.156% in the 6- and 4-wk CCl4-treated groups, respectively, to 2.01±0.28% in the CCl4 group plus GA, compared with 0.38±0.07% in naïve mice (Fig. 1B). GA treatment in the 6-wk CCl4-treated animals therefore significantly ameliorates hepatic fibrosis compared with GA untreated groups (4 and 6 wk), suggesting a real reverse effect on hepatic fibrosis. To determine whether reduced fibrosis in mice treated with GA was associated with decreased stellate cell activation, we evaluated the expression of α-SMA on liver protein extracts by immunoblotting. Indeed, the relative extent of fibrosis correlated closely with α-SMA expression in all groups. As shown in Fig. 1C, reduced fibrosis in the GA-treated mice was associated with diminished expression of α-SMA, indicating decreased stellate cell activation in the tissue. Furthermore, α-SMA mRNA was also determined in livers with quantitative real-time PCR. α-SMA mRNA was increased in the livers of fibrotic animals not treated with GA to 14.4 (SD 3.8)-fold at 6 wk and to 5.2 (SD 2)-fold at 4 wk (P = 0.03). GA treatment, however, significantly decreased α-SMA mRNA expression to 1.5 (SD 1.1)-fold (Fig. 1D) compared with non-GA-treated groups (P = 0.0005 and 0.02, respectively). CYP2E1 activity (Fig. 1E) presented as pm-nitrophenol was significantly lower in the 6-wk CCl4-treated mice [389.7 (SD 214) pmol·min⁻¹·mg protein⁻¹] compared with the control mice [744.2 (SD 83) pmol·min⁻¹·mg protein⁻¹; P = 0.05] because CCl4 is known to lower CYP2E1 levels via radical inactivation and lipid peroxidation (63). GA, however, did not affect CYP2E1 activity [367.5 (SD 3.7) pmol·min⁻¹·mg protein⁻¹; P = 0.01 vs. naïve and P = 0.5 vs. non-GA-treated fibrotic mice], indicating that GA does not alter reactive oxygen species (ROS) production by CCl4.

GA does not affect necroinflammatory liver injury. Serum AST and ALT levels (Fig. 2A) were significantly elevated in both CCl4 groups [104.3 (SD 10.8) and 168 (SD 34.2), respectively, and 95 (SD 14.2) and 179 (SD 26), respectively]
Fig. 1. Tissue sections were stained with Sirius red as described in MATERIALS AND METHODS. A: representative tissue sections. After fibrosis induction by intraperitoneal carbon tetrachloride (CCl4) injections, fibrotic septa are less established in glatiramer acetate (GA)-treated animals (magnification ×10). B: relative fibrosis area, expressed as % of total liver area, was assessed by analyzing 36 Sirius red-stained liver sections per animal. Each field was acquired at ×10 magnification and then analyzed with a computerized Bioquant morphometry system. The relative fibrosis area in the livers of GA-treated animals was lower than that of untreated mice at 4- and 6-wk (W) time points. P values refer to comparisons between indicated groups. C: whole liver protein lysates were extracted, and 30 μg of total proteins was loaded per lane and analyzed for -smooth muscle actin (SMA) and -actin expression. -SMA expression was similar in all tested wells. Decreased -SMA expression was found in lysates from GA-treated mice compared with untreated mice receiving CCl4 (results were reproduced in 3 repeated experiments). D: -SMA mRNA expression in the liver by quantitative real-time-PCR followed a similar pattern. E: cytochrome P-450 2E1 activity was measured by the rate of oxidation of p-nitrophenol (PNP) to p-nitrocatechol in 50 μg of microsomal protein for 20 min at 37°C. *P = 0.05 for CCl4 vs. naive; **P = 0.01 for GA+CCl4 vs. naive; P = not significant for CCl4 vs. GA+CCl4. The findings are representative of at least 4 different experiments with the same number of animals (8–10) in each subgroup.
compared with naive mice [45 (SD 25) and 36 (SD 12)] (P < 0.001) (CCl4 alone and CCl4 with GA). However, there were no appreciable differences between GA-treated and -untreated CCl4 groups (P = 0.26 and P = 0.29 for AST and ALT, respectively). The serum biochemistries were correlated with semiquantitative pathological scoring. The extent of periportal or perisep tal interface hepatitis; confluent necrosis; and focal lytic necrosis, apoptosis, and focal inflammation (C). After fibrosis induction as described in Fig. 1, ALT and AST serum levels and scorings significantly rose in both groups. A repeated experiment showed the same pattern.

Comparative analysis of splenic lymphoid subsets showed a progressive decline in CD4 T cells and an increase in CD8 subsets after fibrosis induction (P < 0.001). Spleen CD8 T cells increased from 8.5% (SD 1.03) in naive animals to 14% (SD 2.7) and 16.2% (SD 2.5) in CCl4 and CCl4 plus GA-treated fibrotic animals, respectively. Liver CD8 T cells increased from 7.6% (SD 0.9) in naive animals to 21.7% (SD 2.4) and 16.4% (SD 1.7) in CCl4 and CCl4 plus GA-treated fibrotic animals, respectively. Compared with nontreated fibrotic mice, GA treatment significantly decreased the number of liver CD8 cells (P < 0.001). In addition to characterization of the CD4 and CD8 subsets, NK cells were also analyzed since they have potential antifibrotic activity. Spleen NK cells decreased from 7.8% (SD 0.6) in naive animals to 3.9% (SD 1.6) in CCl4-treated mice not compared with naive mice [45 (SD 25) and 36 (SD 12)] (P < 0.001) (CCl4 alone and CCl4 with GA). However, there were no appreciable differences between GA-treated and -untreated CCl4 groups (P = 0.26 and P = 0.29 for AST and ALT, respectively).

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Antifibrotic effect of GA is associated with lymphocyte and cytokine modulation. To gain insight into the potential mechanism of GA’s antifibrotic activity, we analyzed splenic and hepatic lymphocytes by FACS. As shown in Fig. 3, splenic CD4 T cells significantly decreased in numbers in both CCl4-treated groups [22.20% (SD 0.99) in naive animals, 13.30% (SD 5.17) in CCl4 alone, and 17.1% (SD 3.5) in CCl4+GA-treated fibrotic animals (Fig. 3A), respectively] (P < 0.001). GA tends to increase CD4 content (P = 0.08) compared with non-GA-treated fibrotic mice. Similarly, intrahepatic CD4 declined after induction with CCl4, from 29.76% (SD 2.7) to 14.07% (SD 2.8) and 16% (SD 0.91) (Fig. 3B), respectively (P < 0.001). No significant changes were observed in liver CD4 subsets between the two fibrotic groups. Both splenic and intrahepatic CD8 cells increased significantly after fibrosis induction (P < 0.001). Spleen CD8 T cells increased from 8.5% (SD 1.03) in naive animals to 14% (SD 2.7) and 16.2% (SD 2.5) in CCl4 and CCl4 plus GA-treated fibrotic animals, respectively. Liver CD8 T cells increased from 7.6% (SD 0.9) in naive animals to 21.7% (SD 2.4) and 16.4% (SD 1.7) in CCl4 and CCl4 plus GA-treated fibrotic animals, respectively. Compared with nontreated fibrotic mice, GA treatment significantly decreased the number of liver CD8 cells (P < 0.001).

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Fig. 2. GA did not affect necroinflammatory liver injury. A: serum aspartate (AST) and alanine (ALT) aminotransferase levels. B: mean necroinflammatory scorings of periportal or perisep tal interface hepatitis (A); confluent necrosis (B); and focal lytic necrosis and apoptosis and focal inflammation (C). After fibrosis induction as described in Fig. 1, ALT and AST serum levels and scorings significantly rose in both groups. A repeated experiment showed the same pattern.

Fig. 3. A: altered splenocyte subsets. Compared with naive animals, fluorescence-activated cell sorting analysis of spleen lymphocytes showed a significant decrease in CD4 T cells and an increase in CD8 subsets after fibrosis induction, both in GA-treated and -nontreated mice (ANOVA, P < 0.001). A significant increase in natural killer (NK) cells was seen in the GA-treated group (P = 0.009). B: altered intrahepatic subsets. Similar to the spleen findings, there was a significant decrease in liver CD4 T cells and an increase in CD8 subsets after fibrosis induction (ANOVA, P < 0.001). The GA-treated group had significantly fewer CD8 cells and higher NK counts compared with the nontreated group (P < 0.001 and P = 0.0007, respectively).
receiving GA ($P < 0.0001$). However, they increased to 7.5\% (SD 2.8) in the CCl4 plus GA-treated group ($P = 0.4$ vs. naive but $P = 0.009$ vs. non-treated fibrotic). Liver NK cells decreased from 9.3\% (SD 1) in naive mice to 5.8\% (SD 0.9) in GA-untreated fibrotic mice ($P < 0.0001$) and increased to 9.9\% (SD 2.1) in the GA-treated fibrotic group ($P = 0.3$ vs. naive but $P = 0.0007$ vs. non-treated fibrotic group). Compared with fibrotic mice, GA treatment significantly increased the number of spleen and liver NK cells.

According to current knowledge, populations of suppressor or Treg cells constitute a pivotal mechanism in regulating immune responses. Treg cells have been implicated in a broad array of immunologic and nonimmunologic diseases (34, 55, 57). Compelling evidence suggests that a delicate equilibrium of controlled self-responsiveness is maintained by a unique population of CD4+CD25+ Treg. The result of this process is the survival of autoreactive lymphocytes representing a variety of high-affinity self-reactive T-cell receptors (28). Mounting evidence indicates that Treg cells participate in the regulation of immune responses to microbial infections by contributing to setting the equilibrium between memory and pathogen elimination (8). Treg cells can be isolated from T-cell infiltrates in ongoing autoimmune diseases (12), and responses to allergens and tumor antigens appear to be controlled by an interplay between Treg and effector T cells (30, 51, 60). CD4+CD25+FoxP3+ Treg cells from tumor-bearing animals impede dendritic cell function by downregulating the activation of the transcription factor NF-kB. The suppression mechanism requires TGF-β and IL-10 (35). In examining whether the regulatory cell (CD4+CD25+FoxP3+) phenotype was associated with GA antifibrotic effect, we observed no significant alterations in splenocytes from two fibrotic groups (Fig. 4A). In the liver, Treg cells significantly decreased in the CCl4 group ($P = 0.02$) compared with the naive group (Fig. 4B). This decrease of Treg cells may account for the increased content of fibrogenic CD8 cells. The inability of Treg cells to regulate the inhibition of CD8 T-cell function was reported to contribute to the initiation of autoimmune liver damage (36). However, only liver CD4+CD25+FoxP3+ T cells correlated well with an antifibrotic effect of GA, because they significantly increased (Fig. 4B). Liver CD4+CD25+FoxP3+ T cells were 18.5\% (SD 2.2) of CD45+ cells in the naive group and significantly decreased to 14.6\% (SD 2.5) ($P = 0.02$) in GA-untreated and increased to 23.2\% (SD 2.8) ($P = 0.007$) in GA-treated fibrotic groups, respectively. Compared with GA-nontreated fibrotic animals, GA treatment significantly increased Treg cells ($P = 0.0006$). An increase in the suppressant Treg cells after GA treatment may explain the direct effect of the Treg cells regarding the suppression of CD8 cells and subsequently the attenuation of hepatic fibrosis.

Since cytokines can either stimulate or inhibit fibrosis (21, 38, 48, 62, 68), we first measured the serum concentrations of the antifibrotic cytokine IFN-γ. As shown in Fig. 5, IFN-γ concentrations in the naive animals were 6 (SD 4) pg/ml and increased to 35.9 (SD 20.7) pg/ml in CCl4-treated mice not receiving GA ($P = 0.001$) and 34.8 (SD 23.6) pg/ml in CCl4 plus GA-treated fibrotic animals ($P = 0.004$). There was no effect of GA on IFN-γ levels in animals treated with CCl4.

We next assayed the levels of the profibrotic cytokine IL-4. Fibrosis induction led to a significant elevation of serum IL-4, from 31.3 (SD 26) to 100 (SD 44) pg/ml ($P = 0.0005$). Importantly, GA treatment markedly reduced IL-4 levels after induction of fibrosis by CCl4 to 55.7 (SD 39.7) pg/ml ($P = 0.03$), suggesting that the antifibrotic effect of GA was partly a result of reduced levels of this profibrotic cytokine (Fig. 5).

The impact of GA was also assessed on serum levels of IL-10, which, in contrast to IL-4, is an antifibrotic cytokine (41, 54). Serum IL-10 concentrations were 31 (SD 41.4) pg/ml in naive mice and 34.2 (SD 16.6) pg/ml after the induction of fibrosis by CCl4 (Fig. 5). GA treatment, however, significantly elevated the IL-10 level in CCl4-treated mice to 63.1 (SD 25.1) pg/ml ($P = 0.02$). Thus an elevation in the IL-10 level in GA-treated mice may also contribute to its antifibrotic activity. HSC apoptosis. HSC apoptosis in freshly isolated cells (Fig. 6A) was significantly increased from 37.2\% (SD 6.4) in naive animals to 49\% (SD 1) in fibrotic animals ($P = 0.005$). GA treatment augmented HSC apoptosis to 53.3\% (SD 5.3) ($P = 0.0005$). Liver CD4+CD25+FoxP3+ T cells were 18.5\% (SD 2.2) of CD45+ cells in the naive group and significantly decreased to 14.6\% (SD 2.5) ($P = 0.02$) in GA-untreated and increased to 23.2\% (SD 2.8) ($P = 0.007$) in GA-treated fibrotic groups, respectively. Compared with GA-nontreated fibrotic animals, GA treatment significantly increased Treg cells ($P = 0.0006$). An increase in the suppressant Treg cells after GA treatment may explain the direct effect of the Treg cells regarding the suppression of CD8 cells and subsequently the attenuation of hepatic fibrosis.

Since cytokines can either stimulate or inhibit fibrosis (21, 38, 48, 62, 68), we first measured the serum concentrations of the antifibrotic cytokine IFN-γ. As shown in Fig. 5, IFN-γ concentrations in the naive animals were 6 (SD 4) pg/ml and increased to 35.9 (SD 20.7) pg/ml in CCl4-treated mice not receiving GA ($P = 0.001$) and 34.8 (SD 23.6) pg/ml in CCl4 plus GA-treated fibrotic animals ($P = 0.004$). There was no effect of GA on IFN-γ levels in animals treated with CCl4.

We next assayed the levels of the profibrotic cytokine IL-4. Fibrosis induction led to a significant elevation of serum IL-4, from 31.3 (SD 26) to 100 (SD 44) pg/ml ($P = 0.0005$). Importantly, GA treatment markedly reduced IL-4 levels after induction of fibrosis by CCl4 to 55.7 (SD 39.7) pg/ml ($P = 0.03$), suggesting that the antifibrotic effect of GA was partly a result of reduced levels of this profibrotic cytokine (Fig. 5).

The impact of GA was also assessed on serum levels of IL-10, which, in contrast to IL-4, is an antifibrotic cytokine (41, 54). Serum IL-10 concentrations were 31 (SD 41.4) pg/ml in naive mice and 34.2 (SD 16.6) pg/ml after the induction of fibrosis by CCl4 (Fig. 5). GA treatment, however, significantly elevated the IL-10 level in CCl4-treated mice to 63.1 (SD 25.1) pg/ml ($P = 0.02$). Thus an elevation in the IL-10 level in GA-treated mice may also contribute to its antifibrotic activity.
0.01 vs. GA-untreated group and $P = 0.0004$ vs. naive). Although cleavage of receptors from enzymes used in cell isolation is possible, this effect should have affected all isolated cells equally, yet differences remained between the different groups. In situ apoptosis of tissue HSC by double staining of $\alpha$-SMA and annexin V confirmed the same findings. The number of double-stained annexin V$^+$ $\alpha$-SMA$^+$ cells (Fig. 6B, left) was markedly increased after fibrosis induction from 3.8 (SD 2.4) to 8 (SD 3.6) x 10$^2$ cells/field ($P = 0.02$), and GA further increased it to 13 (SD 4.3) x 10$^2$ cells/field.
self-limited. On the basis of the available data and current management guidelines, GA is a valuable first-line treatment option for patients with RRMS (46, 62).

The beneficial effect of GA has also been identified in other immune-mediated diseases. For example, GA inhibits type 2 collagen-reactive T-cell clones in animal models of rheumatoid arthritis (16). Furthermore, GA can also inhibit the manifestations of host versus graft rejection (3). In experimental autoimmune uveoretinitis, GA has inhibitory suppressive activity, but in contrast to the models previously described the treated mice failed to produce cytokines such as IL-4, IL-5, and IL-10 (73). The ability of GA to effectively modulate the clinical manifestations and to attenuate the detrimental immune response involved in experimental colitis warrants its further investigation in treating this disease (1).

The effect of GA on fibrosis has not previously been examined. In graft versus host disease, treatment with GA abolished cytotoxic activity toward host targets by induction of the anti-inflammatory response and suppression of inflammatory cytokine secretion (IL-2 and INF-γ) (2). Moreover, GA decreased hepatic fibrosis even less than that seen at week 4 of CCl₄ induction, suggesting a real antifibrotic activity. On the other hand, GA did not alter the CYP2E1 activity between GA-treated and GA-nontreated fibrotic mice. GA, therefore, is not affecting the ROS production by the CCl₄. Our data reveal that GA exerts an antifibrotic effect in the liver, by affecting lymphocyte subsets and also by altering the balance between pro- and antifibrotic cytokines. Notably, there was no significant difference in the inflammatory markers (AST, ALT, or Ishak-Knodell score) between the CCl₄ and the CCl₄ plus GA mice, thus indicating the direct effect of GA on fibrosis apart from its effect on the degree of liver injury. An additional explanation for GA’s lack of an effect on inflammation could be that GA blocks lymphocyte-HSC cell-to-cell interaction.

In experimental models, fibrosis induction is associated with an increase in CD8 subsets and with a decrease in CD4 T cells (23, 54). In mouse models, adoptive transfer of CD8 cells from fibrotic donors to severe combined immunodeficient (SCID) mice induces hepatic fibrosis. In contrast, NK cells are antifibrotic (19), as evidenced by their mediating apoptosis of activated stellate cells. We have investigated the role of NK cells on hepatic fibrogenesis (19). Mouse NK cells express both inhibitory/activating-killing immunoglobulin-related receptors (iKIR/aKIR) specific for class I molecules. Hepatic fibrosis induced by CCl₄ was compared among wild-type male BALB/c mice with combined immunodeficiency (SCID, lacking B and T cells), SCID-Beige mice (lacking B, T, and NK cells), and naive mice. Hepatic fibrosis was significantly increased in all CCl₄-treated groups. SCID-Beige mice had more fibrosis than SCID mice (P < 0.0001), as assessed by morphometry of Sirius red-stained tissue sections. After fibrosis, hepatic NK cells significantly decreased and the aKIR-to-iKIR ratio significantly increased, whereas class I receptor expression on HSC decreased (P < 0.001). Both freshly isolated and in situ HSC displayed a significant increase in cellular apoptosis following induction of fibrosis. In human HSC there was decreased class I receptor expression and increased apoptosis as well, which further increased after blocking of either HSC-related class I or NK-related killer inhibitory receptors. Apoptosis was inhibited by preincubation of NK cells with the granzyme inhibitor 3,4-dichloroisocoumarin. In conclusion, during liver injury NK cells exhibit antifibrotic activity at least

**Fig. 7.** Effect of GA on the activation of human HSC by HCV lymphocytes. HCV peripheral blood lymphocytes were preincubated with either GA or medium, and HSC were incubated alone or with GA for 48 h. As a control, naive lymphocytes were used. HSC activation was then determined by the expression of α-SMA. Preincubation of lymphocytes from hepatitis C virus (HCV) patients with GA resulted in a marked alleviation of HSC activation (top). β-Actin expression was similar under all conditions (bottom). Although the graph shows only 3 patients from each group, all other patients followed the same pattern (data not shown).
in part through stimulation of HSC killing. NK cells kill activated HSC via retinoic acid early inducible 1/NKG2D-dependent and TRAIL-dependent mechanisms, thereby ameliorating liver fibrosis (46).

In this study, GA treatment reduced CD8 in the fibrotic liver of our animal model, with no significant effect on the decreased total CD4 subset. The increase of fibrotic CD8 cells following CCl4 administration may be explained by the marked decrease of the CD4 cells, either total CD4+ or CD4+CD25+Foxp3+ Treg cells. Hepatic Treg cells, however, significantly increased after GA treatment, probably resulting in a new reconstitution of the Treg suppressive effect on CD8 cells. CD8 cells therefore decreased in GA-treated livers, which might explain the alleviation of HSC activation and the reduction of fibrosis. Furthermore, GA increased NK numbers in the liver and spleen after CCl4. In our model, increased NK cells added an additional option for the immunomodulatory effect of GA in fibrosis reduction. Increased HSC apoptosis and TRAIL support the increased killing of HSC by NK cells after GA treatment (40, 46). The mode of action of the drug in humans with MS is believed to involve the induction of glatiramer-reactive regulatory cells, including CD4+ and CD8+ T cells. Glatiramer-reactive Th2 cells are believed to enter the brain and, through cross-reactivity with myelin antigens, produce bystander suppression, anti-inflammatory effects, and neuroprotection (13, 15). Unlike in our study, GA may suppress autoreactive CD4+ effector memory T cells and enhance CD8+ regulatory responses in patients with RRMS (6, 29). Therefore, Treg cells may be considered to be of major importance in the pathogenesis of MS (56, 66). Diverse types of Treg cells, with distinct origins and with disparate and multiple functions, have been described. CD4+CD25+ cells were reported to be involved in the active suppression exerted by the dual altered peptide ligand on myasthenogenic-associated T-cell responses (43). Another study suggests that levels of circulating CD4+CD25+ Treg cells and CD4+CD25(high) Treg cells are not altered in MS patients and are unaffected by substances currently used to modulate the disease, including IFN-β and GA (44).

In addition to its effects on lymphocyte subsets, GA also affected the balance of pro- and antifibrotic cytokines. Specifically, serum IL-10 levels rose, whereas IL-4 levels fell, which were associated with a GA antifibrotic effect. There is a growing body of evidence in other models that GA promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells (27, 66). Heat shock protein 60-stimulated Treg cells were reported to suppress target T cells both by cell-to-cell contact and by secretion of TGF-β and IL-10 (71). Therefore, CD4+CD25+Foxp3+ cells increased by GA might contribute to the enhanced serum IL-10 levels observed in our study. Moreover, GA induces a selective inhibitory effect on the production of dendritic cell-derived inflammatory mediators. After exposure to GA, dendritic cells have an impaired capacity to secrete Th1 polarizing factors, but on the other hand, they induce the effector’s Th2 cells (50) to secrete IL-4 and to enhance the levels of IL-10 (27). In addition, GA contributes to apoptotic elimination of T helper cells (2, 4).

IL-10 is an antifibrotic cytokine. It downregulates fibrogenesis in cultured HSC by decreasing collagen synthesis and increasing the production of collagenase (68). Furthermore, exogenous IL-10 downregulates the expression of metalloproteinase-2 and metalloproteinase-1, both of which are increased after induction of fibrosis (72). The present findings are consistent with our earlier findings (54) in IL-10 transgenic mice, which have reduced fibrosis after exposure to CCl4, associated with suppression of CD8 subsets and IL-4 secretion.

The direct effect of GA on lymphocytes can explain its antifibrotic effect, as shown in the in vitro study (Fig. 6). GA-affected HCV lymphocytes lost their potential to activate HSC. Importantly, those HSC were not directly exposed to the GA itself.

In summary, GA exerts antifibrotic activity in CCl4-induced fibrosis through altered numbers of CD8 and NK subsets. These findings reinforce the growing understanding that subtle alterations in the intrahepatic immunologic milieu can appreciably alter the fibrotic balance in liver injury. Given its very strong safety profile, GA may also merit further evaluation as an antifibrotic agent for patients with chronic fibrosing liver injury.

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