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Association of age dependent liver injury and fibrosis with immune cell populations

Bradley H. Collins¹, Zoie E. Holzknecht¹, Kellie A. Lynn¹, Gregory D. Sempowski², Cathy Choy Smith³, Songling Liu⁴, William Parker¹, and Don C. Rockey⁴

¹Department of Surgery, Duke University Medical Center, Durham, NC

²Department of Medicine, Duke Human Vaccine Institute, Duke University School of Medicine, Durham, NC

³Liver Center Laboratory and Department of Medicine, Duke University Medical Center, Durham, NC

⁴Department of Internal Medicine, Medical University of South Carolina, Charleston, SC

Abstract

Background/Aim—The liver's response to injury is fibrosis, and when chronic, cirrhosis. Age is a critical factor impacting many immune-mediated processes, potentially including the liver's wounding response to injury.

Methods—The effects of age on acute and chronic liver injury were evaluated using a carbon tetrachloride model in mice. Lymphocyte and macrophage populations were assessed by flow cytometry and immunohistochemical analysis.

Results—Acute liver injury was greater in 18-month old (old) mice than in 9-month old (middle aged) mice as judged by changes in aminotransferases. Similarly, 18-month old livers had a significantly greater fibrogenic response to injury than did 9-month old livers after chronic injury (assessed by *coll1a1* mRNA expression, morphometric analysis and hydroxyproline measurement). Interestingly, livers from young mice (6 weeks old) also exhibited an increase in fibrogenesis compared to 9-month old mice, albeit not to the same degree as in old mice. Consistent with a role for macrophages in fibrogenesis, the number of liver macrophages in young and 9-month old mice increased, while in chronically injured 18-month old livers, the number of macrophages was reduced, and was less than in the livers of young and 9-month old injured livers.

Correspondence: Don C. Rockey, MD, Department of Internal Medicine, Medical University of South Carolina, 96 Jonathan Lucas Street, Suite 803, MSC 623, Charleston, SC 29425, rockey@musc.edu, Telephone: 843-792-2914, Fax: 843-792-5265.

Author contributions

BHC, AEH, and DCR were directly involved in study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content. All authors were involved in acquisition of data; analysis and interpretation of data and critical revision of the manuscript for important intellectual content.

Financial Interests

The authors certify that we have no financial arrangements (e.g., consultancies, stock ownership, equity interests, patent-licensing arrangements, research support, major honoraria, etc.) with a company whose product figures prominently in this manuscript or with a company making a competing product.

Conclusions—Our data indicate that the fibrogenic response to injury varies substantially with age, and moreover that macrophage recruitment and dynamics may be an important component in differential age-associated fibrotic disease.

Keywords

extracellular matrix; collagen; cirrhosis; smooth muscle actin; aminotransferase

Introduction

Studies during the 1960's indicated that older male rats (12-months old) developed greater fibrosis than younger rats (1 and 6-months old) long term exposure to carbon tetrachloride (CCl₄) (1, 2). Interestingly, this apparent age effect was limited to male rats, and was not observed in female rats. This difference between male and female animals has been attributed to an age-specific decrease in the antioxidant defense system (3–6), which has been reported to be prominent in male rats but not in female rats or in mice (3). It was also observed that very old rats (30 months) exhibited less acute necrosis induced by chemical injury, but less regeneration following acute injury (5) than did younger rats (2 and 12-months old). A more recent study using male rats demonstrated that 12, 18, and 24-month old rats had greater acute liver injury in response to intraperitoneal injection of ethanol than did younger (3 and 6 month old) rats (7) as judged by serum alanine aminotransferase (ALT) levels. Further, histological analysis revealed that necrosis was greater in the 12 to 24-month old livers compared to younger livers after acute injury. Mechanisms underlying these findings have not been clearly elucidated.

In this study, we hypothesized that intrinsic immune responses in mice may explain divergent responses to injury, and moreover fibrogenesis occurring after said injury. We therefore utilized quantitative measures of liver injury and fibrosis to assess the effect of age on acute and chronic liver injury in mice. We found that while “older” livers appeared to be more susceptible to acute injury than “younger” livers, the chronic fibrotic response to a given injury was profoundly greater in older mice than in younger mice. This profound increase in fibrosis was accompanied by a substantial loss of macrophages in the chronically injured old liver, consistent with the concept that macrophages play a key role in fibrolysis during the recovery phase (8–12). Interestingly, we observed that very young livers (mice aged 6-weeks at the beginning of the seven week study) showed a moderately increased fibrotic response compared to middle aged (9-month old) mice. Unlike the old mice, the increased fibrosis observed in very young mice was not accompanied by a decrease in the number of hepatic macrophages, suggesting that the fibrotic response in very young and old mice may be differentially regulated. However, the increased fibrosis was considerably more pronounced in old mice than in very young mice.

Materials and Methods

Animals

Six week and nine month old male BALB/c mice were purchased from Harlan, Inc. (Indianapolis, IN). Eighteen-month-old male BALB/c mice were purchased from the

National Institute on Aging (Bethesda, MD). Animal care conformed to the standards of the National Society of Medical Research (“Principles of Laboratory Animal Care”) and “The Guide for the Care and Use of Laboratory Animals” (NIH publication No. 86–23, revised 1985). Experiments were approved by the Duke University Institutional Animal Care and Use Committee.

Hepatic injury was induced by carbon tetrachloride (CCl₄) (Sigma Chemical Co., St. Louis, MO) gavage. A mixture of CCl₄ and corn oil (1:1, v) or corn oil alone as a control was given by oral gavage at a dose of 1.5mL/kg body weight once per week for six weeks. Animals were placed under very anesthesia (Tech 3 vaporizer, Harvard Apparatus, Holliston, MA) for the gavage procedure.

Animals were sacrificed by CO₂ inhalation 7 days following the sixth gavage unless otherwise noted. Blood (600 – 1200 µl) was obtained from each animal through the portal vein immediately following sacrifice. The liver was removed and samples were snap-frozen in liquid nitrogen, fixed in 10% neutral buffered formalin (VWR International, West Chester, PA) or assayed immediately using procedures described below.

Lymphocyte isolation

Livers (gall bladders removed) were pressed through a 280 µm screen (Bellco Glass, Inc., Vineland, NJ) and incubated with 0.02% collagenase D and 400 units/ml DNase I in 20 mL Hank's Balanced Salt Solution (HBSS) containing 20 mM HEPES, pH 7.4, at 37°C for 30 minutes with occasional shaking. The slurry was filtered through a 70 µm nylon filter (BD Biosciences-Discovery Labware, Bedford, MA) and centrifuged at 300 × g at 4°C for 5 minutes. The cell pellet was resuspended in HBSS (Gibco BRL, Grand Island, NY) with 33% Percoll (Sigma Chemical Co.) (30 milliliters total) and centrifuged at 800 × g at 4°C for 20 minutes. The upper layer and interface containing parenchymal cells, dead cells and debris was discarded and the pellet containing lymphocytes and red blood cells was washed one time with 50 milliliters HBSS by centrifugation at 300 × g at 4°C for 5 minutes. To lyse red blood cells, the pellet was resuspended in 20 milliliters of freshly prepared ACT buffer (125 mM NH₄Cl and 17 mM Tris, pH 7.3) and incubated at 4°C for 10 min with occasional shaking. After incubation, 30 milliliters of HBSS was added to the cells and lymphocytes were centrifuged at 300 × g at 4°C for 5 minutes. To remove remaining debris the pellet was resuspended in 10 milliliters of HBSS, layered over 3 milliliters lymphocyte separation medium (ICN Biomedicals, Inc., Aurora, OH) and centrifuged at 1500 × g at 4°C for 15 minutes. The resulting interface contained the lymphocyte fraction and was recovered and washed one time with HBSS by centrifugation at 300 × g at 4°C for 5 minutes. Cell concentrations were determined using a Coulter Multisizer IIE (Beckman Coulter, Inc., Brea, CA).

ELISPOT detection of cytokine secretion

Wells of a 96-well MultiScreen-IP plate, Immobilon-P membrane (Millipore Corp., Bedford, MA) were coated with 100 µL of either anti-mouse IFN-γ monoclonal antibody or anti-mouse IL-4 monoclonal antibody (Pierce Biotechnology Inc., Rockford, IL) at 5 µg/mL in phosphate buffer saline (PBS) (Gibco BRL) overnight at 4°C. Wells were washed 3 times

with 200 μ l of PBS and filled with blocking buffer (PBS with 3% bovine serum albumin and 5% heat-inactivated fetal calf serum). Wells were blocked for up to 3 hours at room temperature and washed with ELISPOT medium (RPMI-1640 containing 20 mM HEPES, 10% heat-inactivated fetal calf serum, 1 mM L-glutamine, 100 μ g/ml penicillin, 100 units/ml streptomycin, 8.0 mM NaOH, 1 mM sodium pyruvate, and 1x of each MEM Amino Acids Solution and MEM Non-essential Amino Acids Solution) (Gibco BRL). Isolated mouse lymphocytes (described above) were plated at various concentrations in 200 μ l/well of ELISPOT media containing 10 ng/mL recombinant mouse IL-2 (R&D Systems, Minneapolis, MN) and 10 μ g/ml Concanavalin A (Sigma Chemical Co.) and incubated for 24 to 48 hours at 37°C with 5% CO₂. Wells were washed 3 times with 200 μ l with PBS containing 5% fetal calf serum and 0.05% Tween-20 and 100 μ l/well of either biotin-labeled anti-mouse IFN- γ monoclonal antibody or biotin-labeled anti-mouse IL-4 monoclonal antibody (Pierce Biotechnology) was added at a concentration of 2 μ g/ml in blocking buffer overnight at 4°C. Wells were washed 3 times with 200 μ l PBS containing 0.05% Tween-20 and 100 μ l/well of horseradish peroxidase-conjugated avidin diluted 1:100 in PBS containing 0.05% Tween-20 was incubated for 1–2 hours at room temperature. Wells were washed 4 times with 200 μ l PBS containing 0.05% Tween-20 and 100 μ l/well of AEC chromogen substrate (BD Biosciences-Pharmingen, San Diego, CA) prepared as directed. Wells were developed for 10–30 minutes and then washed several times with tap water to stop the reaction. Images of each well were captured using an ImmunoSpot Image Analyzer system (Resolution Technology Inc., Columbus, OH) and spots were counted manually.

Hydroxyproline measurement

Hydroxyproline content in liver tissue was measured as described (13). In brief, frozen mouse liver tissue was homogenized in 6 N HCl at 20 μ L/mg and hydrolyzed under N₂ gas at 110°C for 16 hours. Hydrolysates were filtered with 0.2 μ m syringe filters and aliquots of 25 μ L were evaporated under vacuum. Sediments were dissolved in 600 μ l of 50% isopropanol. Standards of 4-hydroxyproline (Sigma Chemical Co.) from 0 to 1.6 μ g/mL were made in 50% isopropanol. One hundred microliters of chloramine-T solution (0.56 mL of 15% N-chloro-p-toluenesulfonamide in dH₂O (Sigma Chemical Co.) and 9.44 mL acetate-citrate buffer (0.42 M sodium acetate, 0.13 M sodium citrate, 26 mM citric acid in 39.5% isopropanol, pH 6.0) was added to samples and standards and incubated at room temperature for 10 minutes protected from light. Five hundred microliters of Ehrlich's Reagent (3 milliliters of Ehrlich stock (10 g 4-(dimethylamino)benzaldehyde, 11 milliliters of 60% perchloric acid) (Sigma Chemical Co.) and 8 milliliters of isopropanol) was added to samples and standards and incubated at 50°C for 90 minutes protected from light. Samples and standards were run in triplicate. Sample absorbance was read at 558 nm and the concentration of hydroxyproline was determined by comparison of unknowns with a standard curve.

RNA isolation, reverse transcription and quantitative PCR

Total RNA was isolated from liver tissue using RNeasy silica-gel binding membranes (Qiagen, Valencia, CA) as per the manufacturer's directions. Briefly, frozen liver tissue (10–30 mg) was homogenized and passed through an RNA binding membrane. Genomic DNA was removed using DNase. Total RNA was eluted in dH₂O and quantified by measuring

absorbance at 260 nm. mRNA was reverse transcribed using M-MLV reverse transcriptase (Gibco BRL) and random hexamer primers (Roche Diagnostics Corp., Indianapolis, IN) as per the manufacturer's directions. Real-time PCR was performed with SYBR green detection dye with a Mx3000 Real-Time PCR System (Stratagene, Cedar Creek, TX). Target cDNA was normalized using GAPDH cDNA as a control for each sample and an external standard was used for normalization between runs. Primers for quantitative detection are; mouse GAPDH forward, GTC GTG GAT CTG ACG TGC C, mouse GAPDH reverse, TGC CTG CTT CAC CAC CTT CT; mouse pro- α 1 collagen forward, TGC TTT CTG CCC GGA AGA, mouse pro- α 1 collagen reverse, TGG GTC CTC GAC TCC TAC ATC T; mouse fibronectin ED-A forward, GGG CAA GTT TCC AGG TAC AG, mouse fibronectin ED-A reverse, CAG CTC TGC AGT GTC GTC TT, mouse TGF- β 1 forward, CCT GCA AGA CCA TCG ACA TG, mouse TGF- β 1 reverse, GGA CTG GCG AGC CTT AGT TT. Primers are listed 5' to 3'.

Flow cytometry

Isolated liver lymphocytes (described above) were incubated with mouse serum for 5 minutes at 4°C and the following fluorochrome-conjugated anti-mouse antibodies (BD Biosciences Pharmingen) for 30 minutes at 4°C in PBS with 1% bovine serum albumin: anti-CD3e-FITC and anti-CD3e-APC (clone 145-2c11), anti-CD4-FITC (clone GK1.5), anti-CD4-PE (clone RM4-5), anti-CD8a-Cy-Chrome (clone 53-6.7), anti-CD11b-APC (clone M1/70), anti-CD45R/B220-PE (clone RA3-6B2) and anti-Pan-NK cells-FITC (clone DX5). Optimum concentrations of antibodies and cells were determined experimentally for each pair and appropriate isotype controls were used. Cells were washed with PBS and resuspended at 1×10^6 cells/ml in PBS with 0.4% paraformaldehyde. Multiparameter flow cytometric analysis was performed using a FACS Vantage Cell Sorter and results were analyzed using Cell Quest software (BD Biosciences-Immunocytometry Systems, San Jose, CA) at the Duke Human Vaccine Institute Flow Cytometry Core Facility (Duke University, Durham, NC).

Alanine aminotransferase measurement

Alanine aminotransferase was measured from serum in the Carl Clinical Laboratories (Duke University Medical Center, Durham, NC) by standard methods (14).

Morphometric analysis of hepatic collagen content

Mouse livers fixed in 10% neutral buffered formalin were embedded in paraffin and 4 μ m sections were stained with picrosirius red (which detects collagen) as previously described (15, 16). Briefly, sections were deparaffinized, stained with 0.1% picrosirius red and 0.1% Fast Green FCF in saturated picric acid (Sigma Chemical Co.) for 30 min. Sections were washed briefly in water, dehydrated to 100% xylene and mounted with Permount (VRW International). Collagen was quantified using digital morphometric analysis. A minimum of 10 random fields (20x magnification) per liver section and 2 sections per liver were captured (DS-L1 camera and ACT-1 software, Nikon Inc., Melville, NY), and the amount of picrosirius red stained liver was measured with MetaVue software (Molecular Devices, Sunnyvale, CA). Fibrosis was expressed as the percentage of stained area in each field.

Immunohistochemical staining

For immunohistochemistry of liver sections, ten micron paraffin embedded sections were rehydrated, washed with PBS, heated to 37°C, and then exposed to 1:4 diluted proteinase K (1 mg/mL) for 30 seconds, rinsed with PBS, then after blocking with a dilute milk solution, incubated with anti-F4/80 primary antibody (Abcam, Cambridge, MA), washed in PBS, and antigen detected with a Vectastain ABC Staining Kit as per the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Sections were washed, counterstained with hematoxylin for 1 minute, dehydrated, and mounted (Gel/Mount™; BiØmedia). Images were obtained with a Nikon TE300 microscope (Nikon, Inc., Melville, NY). Control specimens were identical to experimental specimens except they were exposed to irrelevant isotype matched antibody.

Statistics

Results are expressed as mean $\pm$ SD. All experiments were repeated with greater than 6 animals per group. All data were analyzed using a 2-way ANOVA, followed by post-hoc t-tests. Differences were considered significant when $p < 0.05$.

Results

Liver injury following acute injury as a function of age

To determine the effect of age on the extent of liver injury following CCl₄, ALT serum levels in mice of various ages were evaluated (Figure 1). ALT levels in all mice, regardless of age, rose acutely following CCl₄ ($p < 0.05$). ALT levels in old (18-months) mice rose significantly more than levels in 6-week old and 9-month old mice at one-day post- CCl₄ exposure ($p = 0.025$ and 0.0016 , respectively). Interestingly, in the 18-month old mice but not in any of the younger mice, exposure to oil alone over a period of 7 weeks (i.e., no CCl₄; “control” in Figure 1B) led to an increase in ALT levels. Evidence of hepatocellular necrosis 1 and 4 days after administration of CCl₄ paralleled biochemical abnormalities. These data indicate that the older mice are more susceptible to acute liver injury than are younger mice.

Mean serum ALT levels in the youngest mice (6-weeks old at the beginning of the study) rose higher than those of middle-aged (9-month old) mice (Figure 1A). Although the difference in ALT levels between 6-week old and 9-month old mice at one-day post exposure was not significant ($p = 0.070$), the difference in ALT levels at 4 days post exposure was significant ($p = 0.029$).

Serum ALT levels during chronic liver injury were elevated in all mice (Figure 1B) ($p < 0.05$), regardless of age. Eighteen-month old mice and 9-month old mice had higher serum ALT levels than did 6-week old mice ($p = 0.030$ and 0.017 , respectively), although the difference between 18-month old mice and 9-month old mice receiving CCl₄ was not significant. We identified an increase in serum ALT levels in 18-month old mice receiving oil only (control in Figure 1B) compared to younger mice ($p < 0.05$). The observation that oil and CCl₄ exposed 18-month old mice had similar serum ALT levels may reflect the idea that liver necrosis in the CCl₄ mice was limited by advanced fibrosis of those livers. This idea is consistent with the data described in the sections below.

A substantial number of mice died as a result of CCl₄ toxicity. The mortality rate of chronically injured 6-week and 9-month old mice was 27% (4 out of 15) and 25% (5 out of 20), respectively during the course of the study. The mortality rate in 6-week and 9-month controls (oil fed) was 7% (1 out of 14) and 0% (0 out of 16), respectively. Mortality in 18-month old mice chronically exposed to CCl₄ was 50% (7 out of 14) during the course of the study. However, it is likely that some of the 18-month old mice died of causes other than CCl₄ toxicity, since the mortality in the 18-month old group fed oil only (i.e., no CCl₄) was 38% (6 out of 16) during the course of the study.

Fibrosis following liver injury as a function of age

To determine the extent of fibrogenesis in chronically injured livers as a function of age, we assessed type I collagen and fibronectin (cellular) mRNA levels in livers of mice following 6-weeks of CCl₄ induced injury (Figure 2). Type I collagen mRNA (Figure 2A) and fibronectin mRNA (Figure 2B) were more elevated in 18-month old mice than in 6-week old mice ($p = 0.0066$ and 0.0056 respectively). Similarly, type I collagen mRNA and fibronectin mRNAs were more elevated in 6-week old mice than in 9-month old mice ($p = 0.0070$ and 0.020 respectively), although the elevation in type I collagen mRNA and fibronectin mRNA levels in the 18-month old mice far exceeded those of either the 6-week old or the 9-month old mice. These data demonstrate the fibrogenesis is ongoing in the chronically injured liver, and that this process is profoundly greater in the old liver than in younger livers.

We also quantitated fibrosis by measuring hydroxyproline content and by morphometric analysis of picrosirius red stained collagen in injured livers (Figure 3). Fibrosis in the chronically injured livers of old mice was significantly greater ($p < 0.05$) than the injury in younger mice (both 6-week old and 9-months old). Similarly, fibrosis in chronically injured 6-week old livers was significantly greater than in 9-month old livers.

Immune milieu associated with liver injury as a function of age

We hypothesized that age-associated differences in the susceptibility and response to chronic liver injury may be due, at least in part, to age-dependent differences in immune status. As part of our evaluation of the immune milieu following liver injury, we evaluated the levels of IFN- γ producing cells, IL-4 producing cells, and TGF- β mRNA in acutely and chronically injured livers (Figure 4). Substantial age-associated differences in these cytokines were observed in both acutely and chronically injured livers. The levels of IFN- γ producing cells increased in 6-week old and 18-month old acutely injured mouse livers but not in 9-month old mouse livers. Chronic injury, on the other hand, was associated with increased levels of IFN- γ producing cells in 18-month old mice only, with significant ($p < 0.05$) decreases (relative to the uninjured liver of the same age) of IFN- γ producing cells in 6-week old and 9-month old mice. Similarly, levels of IL-4 producing cells varied as a function of the type of injury and the age of the injured mouse (Figure 4). For example, levels of IL-4 secreting cells increased dramatically in chronically injured 6-week old livers, to a lesser extent in chronically injured 18-month old livers, and not at all in 9-month old chronically injured livers. TGF- β mRNA levels were significantly increased in chronically injured 6-week old livers ($p < 0.05$), but not in chronically injured 9-month old livers. Although levels of TGF- β mRNA did not increase in the chronically injured 18-month old

livers compared to the normal 18-month old liver, levels of TGF- β mRNA were elevated in all 18-month old livers compared to the 6-week and 9-month animals ($p < 0.02$). Since TGF- β is an important cytokine involved in fibrosis, these data are consistent with the idea that the age-dependent immune milieu may be associated with the increased fibrosis observed in old livers. Further, these data suggest that, to the extent that fibrotic injury processes are immune mediated, those processes are fundamentally distinct in very young (6-week) mice compared to old (18-month) mice.

To further explore of the age-dependent immune milieu associated with chronic liver injury, the number of total cells, CD3+/CD4+ cells, CD3+/CD8+ cells, B-cells, NK cells, and macrophages was evaluated in normal mice and in mice with acute and with chronic liver injury. A number of significant ($p < 0.05$) changes in the immune cell populations were observed. Transient increases in the CD3+CD8+ cell, NK cell and B cell populations were observed following acute liver injury across all age groups (Figure 5), but no significant increases of these populations were identified in chronically injured livers (Figure 6). This could suggest that variations in lymphocyte responses to acute injury as a function of age trigger later reactions involved in chronic injury. Increases in the CD3+CD4+ population were observed in 18-month old mice following chronic liver injury, while a decrease was observed in 6-week old and 9-month old mice with chronic liver injury (Figure 6).

Since macrophages appear to play key roles in both fibrogenesis and fibrolysis in the liver, we explored macrophages in each acutely and chronically injured livers. The level of macrophages in normal 18-month old livers was greater than that in either 6-week old or 9-month old livers (Figure 7). Changes in the levels of CD11 positive cells following chronic injury were dramatically age-dependent: A two-fold increase in CD11 positive cells was observed following chronic injury of 6-week old and 9-month old mice ($p = 0.0045$ and 0.0135 , respectively, compared to normal). In contrast, a two-fold decrease in CD11 positive cells following chronic injury to 18-month old livers was observed ($p = 0.0009$ compared to normal). Liver macrophages, assessed by F4/80 immunostaining of chronically injured livers revealed an increase in macrophages following chronic injury of 6-week old and 9-month old mice, and a substantial decrease in macrophages in 18 month old mice (Figure 8).

Discussion

The immune system appears to play a key role in both acute and chronic liver injury. Macrophages, B and T cells, and perhaps even other cells may be linked to the fibrogenic and fibrolytic processes (9, 17–24). For example, macrophages can secrete PDGF and TGF- β 1, which can stimulate hepatic stellate cells and myofibroblasts, inducing a fibrogenic phenotype in those cells (9, 25–27). Additionally, macrophages may play a role in the fibrolytic process by secreting TRAIL, TNF- α , and other pro-apoptotic mediators (9, 28, 29), thus directly inducing apoptosis of hepatic stellate cells, which in turn leads to increased fibrolysis through downregulation of extracellular matrix production and TIMPs (30). Given the important role of the immune system in fibrosis, it is reasonable to hypothesize that changes in the immune system as a function of age play a key role in the fibrotic response to injury exhibited by old livers.

Our data demonstrate that (a) old livers are more susceptible to injury and that (b) there is more fibrosis in old livers following a given injury. Following exposure to CCl₄, acute liver injury as measured by serum ALT levels was approximately twice as high in 18-month old mice as in 9-month old mice. However, chronically injured livers of 18-month old mice had nearly 50-fold greater collagen mRNA and 10-fold greater fibronectin mRNA expression than livers of comparable 9-month old mice, indicating substantial, ongoing fibrogenesis in the chronically injured, old liver. Direct measures of fibrosis (hydroxyproline quantification and picrosirius red staining) indicated a four to five fold increase in fibrosis of 18-month old chronically injured livers compared to livers from comparably treated 9-month old mice.

Taken together, these results demonstrate the profound propensity for the “old” liver to become fibrotic in the face of injury. Although old livers appear to be somewhat more susceptible to injury, the most remarkable feature of the old liver appears to be the dramatic increase in fibrosis. In the experimental model used, acute injury in old livers was greater than that in younger livers by a factor of two, whereas the fibrosis observed after chronic injury was greater by a factor of four to five. These data may provide a potential explanation for the clinical observation that older livers exposed to hepatitis C develop fibrosis more rapidly than “younger” livers (31, 32), and further that advanced donor liver age in liver transplantation for hepatitis C is associated with more rapid fibrosis progression (33–36).

Interestingly, very young livers appeared to also be more susceptible to a fibrogenic response than did middle aged livers. Of note is the fact that, although the youngest mice used in this study were relatively young (6-weeks old) at the onset of the study, their age had more than doubled by the completion of the study. Thus, studies on even younger animals could demonstrate even greater fibrosis in response to chronic liver injury than was observed in this study. However, such studies would necessarily be conducted on animals other than mice because of the technical difficulty associated with study of mice less than 6-weeks old.

Given the evolving body of literature surrounding the role of macrophages in fibrosis (37–42), our study raises a number of further questions. Previous data suggest that the role of macrophages in fibrogenesis is complex, with the potential for macrophages to stimulate (37–39), as well as ameliorate fibrosis (39–42). The possibility that macrophages secrete metalloproteases important in fibrolysis is an attractive mechanistic explanation for the observations reported here (39–41). Therefore, our work further highlights several new lines of investigation relevant to the further understanding of the role of age-associated differences in macrophages (e.g. perhaps in macrophage-depleted animals). *Ex vivo* examination of the activity of macrophages derived from young and old mice would also be attractive.

In summary, the effect of age on the fibrotic liver response is paralleled by numerous differences in the immune systems of young and old mice. We observed substantial age-dependent differences in cytokines involved in the fibrotic response, including TGF- β , as well as changes in the number of cellular components, including T-cells, NK cells, CD11 positive cells, and F4/80 macrophages. Perhaps most striking was the loss of macrophages in the chronically injured old liver - in contrast to the increases observed in younger livers. These findings lead us to speculate that in chronic injury in the aged liver, macrophages

participating in the initial liver injury response may be involved in fibrogenesis, whereas macrophages participating in the repair process following injury may be critical for fibrolysis. Thus, the data raise the possibility that there is (a) increased fibrogenesis at early stages of chronic injury, with a higher number of pro-fibrogenic macrophages in older livers compared to younger livers, and (b) decreased fibrolysis at later time points in chronic injury, with lower numbers of pro-fibrolytic macrophages in older livers compared to younger livers.

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Abbreviations

CCl ₄	carbon tetrachloride
IFN- γ	interferon- γ

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Figure 1. Serum alanine aminotransferase levels after acute and chronic injury in different aged mice

Mice aged 6-weeks, 9-months or 18-months of age were exposed to CCl_4 as in Methods. In (A), CCl_4 was administered once and serum was collected 1, 4 or 7 days after a single dose. Groups included untreated (**normal**) mice, mice which received 1 dose of corn oil (**control**) at 1.5ml/kg and mice which received 1 dose of CCl_4 (**injured**) diluted in corn oil (1:1, v) at 1.5 ml/kg body weight (n = 8 in each group). In (B), groups included untreated (**normal**) mice, mice which received 1 dose per week of corn oil (**control**) at 1.5ml/kg for 6-weeks at 7 day intervals and mice which received 1 dose per week of CCl_4 (**injured**) diluted in corn oil (1:1, v) at 1.5ml/kg for 6-weeks. Alanine aminotransferase levels were measured in serum from blood drawn 7 days after the 6th dose (n = 6 in each group). The normal, untreated, mice were the same mice in both graphs. Data in each graph were analyzed separately as in Methods, and significant *p*-values are shown.

Figure 2. Detection of type I collagen and fibronectin mRNA in different aged mice

Exposure to CCl_4 began at 6-weeks, 9-months or 18-months of age as in Figure 1. Groups included untreated (**normal**) mice, mice which received 1 dose per week of corn oil (**control**) at 1.5ml/kg for 6-weeks and mice which received 1 dose per week of CCl_4 (**injured**) diluted in corn oil (1:1, v) at 1.5ml/kg for 6-weeks. Livers were harvested on the 7th day after the 6th dose. Total RNA was extracted and subjected to RT-PCR as in Methods. Type I collagen (**A**) and EDA fibronectin (**B**) mRNAs were measured ($n = 8$ for each group). Data in each graph were analyzed separately using a 2-way ANOVA as in Methods and post-hoc t-tests were run comparing peak responses as a function of age, and significant p -values are shown.

Figure 3. Collagen content after chronic injury in livers of different aged mice

Mice were as in Figure 2. In (A), hydroxyproline was measured as in Methods ($n = 6$ in all groups). In (B), mouse livers were subjected to morphometric analysis of collagen content as in Methods ($n = 9$ in each group). In (C, D and E) are shown representative picrosirius red stained liver sections from 6-week, 9-month and 18-month old mice respectively. The bar shown in (C) represents 100 microns. Data in each graph were analyzed separately using a 2-way ANOVA as in Methods, and significant p-values are shown..

Figure 4. Cytokine production by liver lymphocytes of different aged mice after acute and chronic injury

In (A, C, and E), CCl₄ was administered to mice aged 6-weeks, 9-months or 18-months and livers were harvested 4 or 7 days after a single dose. Groups included untreated (**normal**) mice, mice which received 1 dose of corn oil (**control**) at 1.5ml/kg and mice which received 1 dose of CCl₄ (**injured**) diluted in corn oil (1:1, v) at 1.5 ml/kg body weight (n = 6 in each group). In (B, D and F), exposure to CCl₄ began at 6-weeks, 9-months or 18-months of age. Groups included untreated (**normal**) mice, mice which received 1 dose per week of corn oil (**control**) at 1.5ml/kg for 6-weeks and mice which received 1 dose per week of CCl₄ (**injured**) diluted in corn oil (1:1, v) at 1.5ml/kg for 6-weeks. Livers were harvested on the 7th day after the 6th dose (for B and D n = 9 in each group, for F n = 5 in each group). In (A and B), lymphocytes were isolated from livers taken 4 days and 7 days after treatment. In (C and D), lymphocytes were isolated from livers harvested 7 days after the 6th dose of CCl₄

and ELISPOT assays to detect IFN- γ (A/B) or to detect or IL-4 (C/D) were performed as in Methods. In (E) and (F), liver tissue was harvested 4 days (E) and 7 (F) days after the first or last dose of CCl₄ was administered, respectively and TGF- β mRNA was measured by RT-PCR as in Methods, normalized to the level of GAPDH mRNA; data are presented graphically. In (F), liver tissue was harvested 7 days after the 6th dose and TGF- β mRNA was measured as in (E). Data in each graph were analyzed separately using a 2-way ANOVA and were found to be significantly different ($p < 0.05$) for both age and treatment group or for the interaction between the two, with the exception of the data in graph 4F, where age but not treatment affected the result. Post-hoc t-tests were run comparing changes associated with peak responses at a given age, and significant p -values are shown.

Figure 5. Lymphocyte populations after acute injury in different aged mice

CCl₄ was given to mice aged 6-weeks, 9-months, or 18-months as in Figure 1. Groups included untreated (**normal**) mice, mice that received 1 dose of corn oil (**control**) at 1.5 mL/kg and mice that received 1 dose of CCl₄ (**injured**) diluted in corn oil (1:1, v) at 1.5 mL/kg body weight. Liver tissue was harvested and cells were isolated either 4 or 7 days after CCl₄; in (A), total lymphocytes were measured by flow cytometry as in Methods (n = 6 in each group). In (B–E), specific lymphocyte populations were characterized with specific antibodies by flow cytometry as in Methods (n = 6 in each group). Data in each graph were analyzed separately using a 2-way ANOVA as in Methods, and significant *p*-values are shown.

Figure 6. Lymphocyte populations after chronic injury in different aged mice

Exposure to CCl₄ began at 6-weeks, 9-months or 18-months of age. Groups included untreated (**normal**) mice, mice which received 1 dose per week of corn oil (**control**) at 1.5ml/kg for 6-weeks and mice which received 1 dose per week of CCl₄ (**injured**) diluted in corn oil (1:1, v) at 1.5ml/kg for 6-weeks. Liver tissue was harvested and cells were isolated on the 7th day after the 6th dose of CCl₄. In (A), total lymphocytes were measured by flow cytometry as in Methods (n = 6 in each group). In (B–E), specific lymphocyte populations were characterized with specific antibodies by flow cytometry as in Methods (n = 6 in each

group). Data in each graph were analyzed separately using a 2-way ANOVA as in Methods, and significant p -values are shown.

Figure 7. CD11b populations in the liver after acute and chronic injury of different aged mice
 In (A), mice aged 6-weeks, 9-months or 18-months were given one dose of CCl₄. Macrophages were isolated with flow cytometry as in Methods (using anti-mouse CD11b-APC-conjugated antibody (clone M1/70)) from livers 4 and 7 days after CCl₄ (n = 6 in each group). In (B), macrophages were isolated with anti-mouse CD11b-APC-conjugated antibody as in (A) in mice aged 6-weeks, 9-months or 18-months who were given CCl₄ or corn oil weekly for 6-weeks (n = 6 in each group). Data in each graph were analyzed separately using a 2-way ANOVA and the data in graph 7A were found to be significantly different ($p < 0.05$) for treatment group but not age, whereas the data in graph 7B were significantly different ($p < 0.05$) for both age and treatment group and for the interaction between the two. Post-hoc t-tests compared changes associated with peak responses at a given age, and significant p -values are shown.

Figure 8.

F4/80 positive cells are reduced in chronically injured old livers.

Immunohistochemistry was performed as in Methods to detect F4/80 positive cells in chronically injured, different aged, livers as in Figure 1. F4/80 positive cells were counted in a blinded fashion (10 random fields for each specimen), and quantitative data presented graphically (n = 3 each group); significant *p*-values are shown.