Table 4. Diagnostic Performance of Biochemical Markers and Scores by Stage of Fibrosis

	No Significant Fibrosis (F0-1) vs. Significant Fibrosis (F2-4)					No Severe Fibrosis (F0-2) vs. Severe Fibrosis (F3-4)					No Cirrhosis (F0-3) vs. Cirrhosis (F4)				
	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)
LecT-Hepa	0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	0.882 (0.830-0.949)	83.3	80	59.7	93.1	0.929 (0.896-0.976)	84.6	88.5	58.8	97.2
HA	0.756 (0.684-0.827)	68.1	78.7	77.8	69.6	0.839 (0.771-0.908)	77.1	82.2	61	90.3	0.866 (0.790-0.942)	88.5	75.8	37.3	96.8
TIMP1	0.697 (0.619-0.774)	65.9	71.9	70.4	60.7	0.753 (0.665-0.841)	75	76.3	53	88.9	0.783 (0.710-0.887)	80.8	74.5	27.8	94.6
Platelets	0.729 (0.656-0.803)	78.7	61.9	68.5	73.5	0.821 (0.751-0.891)	81.3	70.4	49.4	91.3	0.851 (0.785-0.918)	84.6	70.7	32.3	95.8
APRI	0.777 (0.709-0.844)	71.3	71.9	72.2	68.8	0.840 (0.780-0.900)	81.3	72.6	50.6	91.5	0.787 (0.703-0.871)	76.9	68.2	27.9	93.9
Fib-4	0.747 (0.671-0.818)	65.9	76.4	74.7	68	0.811 (0.733-0.889)	77.1	73.3	50	89.2	0.856 (0.788-0.924)	73.1	80.9	37.5	94.1
Forns	0.783 (0.716-0.852)	73.4	77.5	77.5	73.4	0.861 (0.802-0.920)	81.3	71.1	50	91.4	0.887 (0.831-0.943)	84.6	75.2	36.1	96.7
Zeng	0.791 (0.723-0.858)	82.9	70.7	75	79.7	0.863 (0.799-0.925)	81.3	79.8	59.5	92.8	0.853 (0.783-0.933)	92.3	73.9	36.9	98.3

AUC, area under the ROC curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive values; NPV, negative predictive values.

DSA) on AGP are normalized by an internal standard lectin (DSA), LecT-Hepa is not influenced by the amount of AGP. We confirmed that the use of this lectin set was statistically superior to the previously selected lectins (AAL and RCA120).

This triplex-sandwich immunoassay employing DSA/MAL/AOL lectins and an anti-AGP antibody from the lectin microarray has already been converted to a fully automated immunoassay analyzer (HISCL-2000i) for clinical use.[15] Pretreatment requires 3 hours, and quantifying the two glyco-parameters for the LecT-Hepa to use this automated analyzer takes 17 minutes. Currently, we can obtain data from LecT-Hepa to predict liver fibrosis on the same day of blood sample collection. This simple and reliable glycomarker may be suitable for clinical use, and may substitute for liver biopsy in some cases.

We are confident that our study samples are representative of most patients. The AUC scores for distinguishing significant fibrosis, severe fibrosis, and cirrhosis by APRI, HA, Fib-4 index, Forns index, and Zeng's score were not significantly different from those in previous studies.[11,27,28] Every serum sample in this study was obtained from a patient immediately before or no more than 2 months after liver biopsy. As many serum samples as possible were collected from each liver center to eliminate a selection bias in any center. Since we could not perform liver biopsy on the patients who had a tendency to develop hemorrhages, fewer samples of severe fibrosis and cirrhosis were collected than those of milder fibrosis. In fact, the population of fibrosis staging in this study was similar to that of a previous, large prospective study evaluating noninvasive fibrosis markers.[29] In addition, we did not include patients with obvious decompensated cirrhosis. This is because inclusion of patients with severe liver disease would have artificially improved the predictive values of the logistic function. On the other hand, we included many patients with mild histological features (48.6% with F0-1). Sampling variation poses potential difficulties, especially in the early stages of disease, when fibrosis might be unevenly distributed.

There are several advantages in using reliable noninvasive markers for assessing liver fibrosis. First, they can be used to accurately determine the appropriate time for initiating IFN treatment in CHC patients. These markers can also help monitor and assess the therapeutic efficacy of IFN treatment in improving liver function in cases of liver fibrosis and cirrhosis. Finally, these markers will be essential in the development of new, antifibrotic treatments. Recently, many directed or targeted therapies against liver fibrosis,

such as anti-transforming growth factor beta and anti-tumor necrosis factor alpha compounds have been developed.[30,31] To evaluate these new drugs, reliable and simple noninvasive fibrosis markers are needed. LecT-Hepa appears to be one of the most prominent candidates to serve as a marker for developing antifibrotic drugs.

In conclusion, both glyco-parameters (AOL/DSA and MAL/DSA) using lectins in a bedside, clinical chemical analyzer succeeded in the quantification of the progression of liver fibrosis. Using LecT-Hepa, the combination score of both AOL/DSA and MAL/DSA is a reliable method for determining fibrosis staging and can be a good substitute for liver biopsy.

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Interferon-Gamma–Mediated Tissue Factor Expression Contributes to T-Cell-Mediated Hepatitis Through Induction of Hypercoagulation in Mice

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Concanavalin A (Con A) treatment induces severe hepatitis in mice in a manner dependent on T cells, interferon (IFN)-gamma, and tumor necrosis factor (TNF). Treatment with the anticoagulant heparin protects against hepatitis, despite healthy production of IFN-γ and TNF. Here, we investigated molecular and cellular mechanisms for hypercoagulation-mediated hepatitis. After Con A challenge, liver of wild-type (WT) mice showed prompt induction of *Ifnγ* and *Tnf*, followed by messenger RNA expression of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1), which initiate blood coagulation and inhibit clot lysis, respectively. Mice developed dense intrahepatic fibrin deposition and massive liver necrosis. In contrast, *Ifnγ*$^{-/-}$ mice and *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice neither induced *Pai1* or *Tf* nor developed hepatitis. In WT mice TF blockade with an anti-TF monoclonal antibody protected against Con A–induced hepatitis, whereas *Pai1*$^{-/-}$ mice were not protected. Both hepatic macrophages and sinusoidal endothelial cells (ECs) expressed *Tf* after Con A challenge. Macrophage-depleted WT mice reconstituted with hematopoietic cells, including macrophages deficient in signal transducer and activator of transcription-1 (STAT1) essential for IFN-γ signaling, exhibited substantial reduction of hepatic *Tf* and of liver injuries. This was also true for macrophage-depleted *Stat1*$^{-/-}$ mice reconstituted with WT macrophages. Exogenous IFN-γ and TNF rendered T-cell-null, Con A–resistant mice deficient in recombination-activating gene 2, highly susceptible to Con A–induced liver injury involving TF. *Conclusions*: Collectively, these results strongly suggest that proinflammatory signals elicited by IFN-γ, TNF, and Con A in both hepatic macrophages and sinusoidal ECs are necessary and sufficient for the development of hypercoagulation-mediated hepatitis. (HEPATOLOGY 2013;57:362-372)

Concanavalin A (Con A)-induced hepatitis is a well-characterized, representative mouse model of T-cell-mediated acute liver failure.[1] After Con A challenge, mice show elevation of circulating proinflammatory cytokine levels, subsequently resulting in massive liver necrosis with dense infiltration of leukocytes. Because interferon (IFN)-γ or tumor necrosis factor (TNF) blockade and gene depletion of *Ifnγ* or

Abbreviations: Abs, antibodies; ALI, acute liver injury; ALT, alanine aminotransferase; B6, C57BL/6; BM, bone marrow; Ccl2, CC chemokine ligand 2 gene clodronate liposome, liposome-encapsulated dichloromethylene bis-phosphonate; Con A, concanavalin A; ECs, endothelial cells; H&E, hematoxylin and eosin; IFN, interferon; IFNAR1, IFN-α receptor 1; IgG, immunoglobulin G; IHC, immunohistochemistry; Il6, interleukin-6 gene; Il1β, interleukin-1β gene; IP, intraperitoneal; IV, intravenously; KO, knockout; Mø, macrophages; mAb, monoclonal antibody; mRNA, messenger RNA; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time reverse-transcriptase polymerase chain reaction; RAG2, recombination-activating gene 2; rIFN-γ, recombinant IFN-γ; rRNA, ribosomal RNA; rTNF, recombinant TNF; SC, subcutaneously; SEC, sinusoidal endothelial cells; STAT1, signal transducer and activator of transcription 1; TAT, thrombin antithrombin III complex; TF, tissue factor; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphage nick-end labeling; WT, wild type.

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**These authors equally contributed to the present study.*

Tnf rescues mice from Con A–induced hepatitis,[2,3] IFN-γ and TNF are convincingly regarded as the cytokines necessary for the development of this type of liver injury. Thus, one may assume that endogenous IFN-γ and TNF initiate both the hepatic inflammatory responses and the liver parenchymal cell death.[4] However, as previously reported, massive liver necrosis is accompanied by severe thrombocytopenia and intrahepatic hemostasis, and heparin pretreatment substantially protects against liver injury without down-regulating the production of IFN-γ and TNF.[2] This may imply that microcirculatory disturbances resulting from hepatic thrombosis contribute to liver injury independent of IFN-γ/TNF-mediated hepatic inflammation and hepatocytotoxicity. Alternatively, IFN-γ and/or TNF might be causative for hepatic thrombosis, perhaps by inducing procoagulant activity within the liver. Thus, it is important to elucidate whether and how IFN-γ and/or TNF contribute to the hepatic hypercoagulation and whether IFN-γ and/or TNF are sufficient to trigger these pathological changes.

Tissue factor (TF) is a transmembrane cofactor for the coagulation factor, VIIa, and is constitutively expressed in the blood vessel wall and its expression is induced by various mediators in several cell types including macrophage (Mø) and endothelial cells (ECs).[5-7] Endothelial damage or TF expression on circulating monocytes/macrophages brings TF in contact with circulating factor VIIa to initiate the blood coagulation cascade, which eventually results in the activation of prothrombin, leading to fibrin formation and platelet activation. The coagulation system is tightly regulated by the fibrinolytic system, which comprises plasminogen, the tissue-type plasminogen activator (tPA), and its inhibitor, plasminogen activator inhibitor-1 (PAI-1).[8,9] *Pai1*$^{-/-}$ mice have been reported to be resistant to alcohol-induced or cholestatic liver injuries.[10-12] Therefore, it is possible that PAI-1 as well as TF may play a role in coagulation-mediated liver injuries.

In this study, we investigated the mechanisms by which Con A treatment induces the prothrombotic state. We found strong induction of hepatic *Tf* and *Pai1* expressions, dense hepatic fibrin deposits, and massive liver necrosis in Con A–treated wild-type (WT) mice, but not in *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice. TF blockade protected WT mice from the intrahepatic fibrin deposition and resultant hepatitis. Both hepatic macrophages (Mø) and sinusoidal ECs (SECs) expressed *Tf* in Con A–challenged WT mice. IFN-γ signaling was crucial for *Tf* induction in both these cell types. Con A–resistant mice that have Mø and SECs, but not T cells, became highly susceptible to Con A when treated simultaneously with IFN-γ and TNF. Collectively, these results indicate that IFN-γ-, TNF-, and Con A–activated signaling pathways in hepatic Mø and SECs are necessary and sufficient for the development of intrahepatic hemostasis-mediated massive liver injuries.

Materials and Methods

Reagents. Con A was purchased from J-Oil Mills (Tokyo, Japan). Neutralizing rat antimouse TF monoclonal antibody (mAb) (1H1) was described elsewhere.[13] Purified rat immunoglobulin G (IgG) was purchased from Beckman Coulter (Fullerton, CA). Recombinant murine IFN-γ and TNF were from PeproTech (Rocky Hill, NJ). Liposome-encapsulated dichloromethylene bis-phosphonate (clodronate liposome) and phosphate-buffered saline (PBS) liposomes were prepared as described previously.[14,15]

Induction of Acute Hepatitis. Con A was administered to mice (20 mg/kg) through a tail vein.[2] In some experiments, mice received Con A intravenously (IV), promptly followed by intraperitoneal (IP) treatment with recombinant IFN-γ (rIFN-γ; 500 ng) and recombinant TNF (rTNF; 500 ng). In some experiments, mice were treated IP with neutralizing anti-TF mAb, 1H1, or subcutaneously (SC) with heparin (5,000 U/kg) 30 minutes before Con A challenge. At various time points after challenge, plasma and liver specimens were sampled.[16] Plasma alanine aminotransferase (ALT) and aspartate aminotransferase levels were measured (SRL, Osaka, Japan).

Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction. We performed quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR), as shown in the Supporting Materials. RNA content was normalized based on amplification of 18S ribosomal RNA (rRNA) (18S).[17] Change folds = normalized data of experimental sample/normalized data of control.

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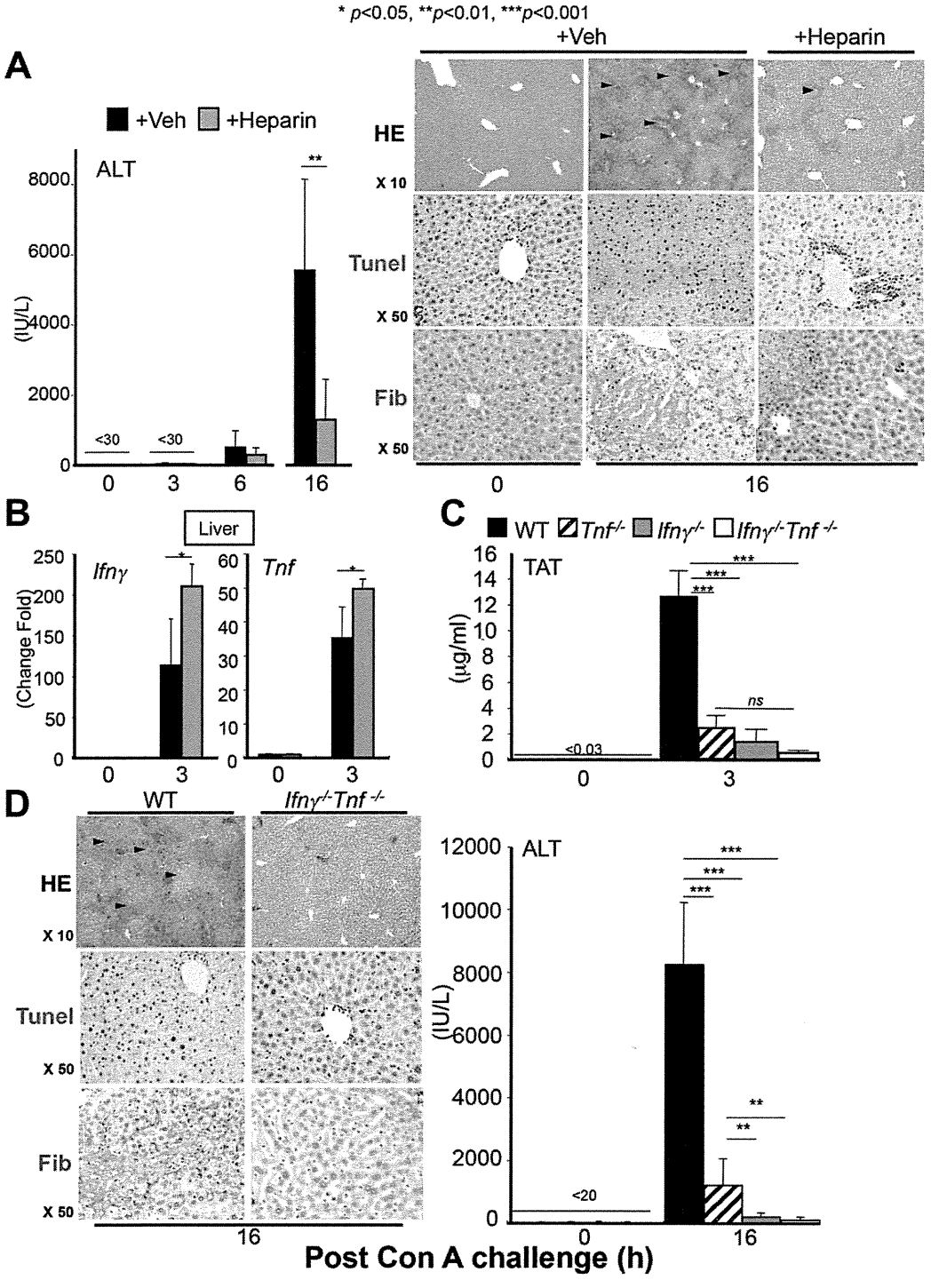

Fig. 1. Both IFN-γ and TNF are necessary for the development of thrombus-associated ALI. (A and B) WT mice were treated SC with heparin (red columns) or vehicle (Veh, closed columns), then with Con A. At the indicated time point, plasma and liver specimens were sampled for measurement of ALT (A) and hepatic *Ifnγ* (B) and *Tnf* (B), respectively. Fold increase of mRNA expression was calculated after normalization to 18S (B). Histological (H&E) and immunohistological study for TUNEL and fibrin deposition (A) were also performed. (C and D) WT (closed bars), $Tnf^{-/-}$ (hatched bars), $Ifn\gamma^{-/-}$ (gray bars), and $Ifn\gamma^{-/-}Tnf^{-/-}$ mice (open bars) were treated with Con A. At the indicated time point, plasma and liver specimens were sampled for measurement of TAT (C) and histological/immunohistological study, as shown in (B), respectively. Representative data are shown (A and D left panels). Original magnification, ×10 (A and D, left upper panels) and ×50 (A and D, left lower panels). Arrowheads indicated necrotic area.

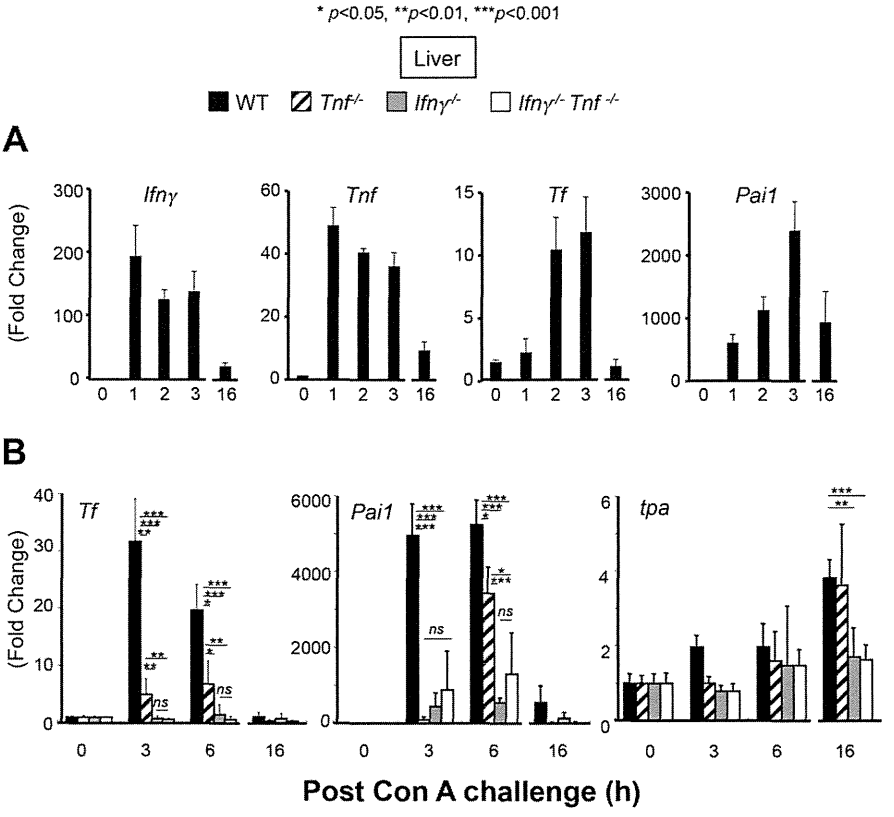

Fig. 2. Requirement of IFN-γ and TNF for the induction of hepatic *Tf* and *Pai1*. WT (closed bars), $Tnf^{-/-}$ (hatched bars), $Ifn\gamma^{-/-}$ (gray bars), or $Ifn\gamma^{-/-}Tnf^{-/-}$ mice (open bars) were challenged IV with Con A, and their liver specimens were sampled for measurement of mRNA expression levels of IFN-γ (A), TNF (B), TF (A and B), PAI-1 (A and B), and tPA (B) by real-time qRT-PCR. Fold increase of mRNA expression was calculated after normalization to 18S.

Assay for Thrombin Antithrombin III Complex. Plasma levels of thrombin antithrombin III complex (TAT) were measured by commercially available kits for TAT (Enzyme Research Laboratories, South Bend, IN).[16]

Preparation of Liver Cells. Hepatic nonparenchymal cells from 3 mice were pooled.[15] $CD11b^+$ hepatic Mø and $CD146^+$ SECs were then enriched by magnetic-activated cell sorting (Miltenyi Biotec GmbH, Cologne, Germany) using anti-CD11b and anti-CD146 microbeads (Miltenyi Biotec), according to the manufacture's instruction, respectively. Stellate cells and liver parenchymal cells were prepared as described.[18,19]

Histological and Immunochemical Analyses. Formalin-fixed tissue sections were stained with hematoxylin and eosin (H&E).[2]

For detection of fibrin deposition, livers were perfused through a portal vein with PBS,[2] and liver specimens were rapidly sampled, fixed in 10% zinc fixative (Becton Dickinson, San Diego CA), and embedded in paraffin. Tissue sections were incubated overnight with rabbit antimouse fibrinogen antiserum (1:5,000) (Molecular Innovations, Inc., Novi, MI), followed by treatment with the rabbit Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, CA). Antigen-antibody (Ab) complexes were detected by using a DAB Substrate Kit (Vector Laboratories). Formalin-fixed liver sections were analyzed for apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphage nick-end labeling (TUNEL) assay.[20]

In Vivo *Depletion of Mø.* Mø were depleted by the IV injection of clodronate liposome, as described previously.[14]

Mouse Reconstitution. To abolish irradiation-resistant Mø, we injected IV clodronate liposome into host mice and, 2 days later, irradiated them, followed by transfer of donor bone marrow (BM) cells.[21,22] CD45.1 WT mice were transferred with CD45.2 WT or CD45.2 $Stat1^{-/-}$ BM cells, and CD45.2 $Stat1^{-/-}$ mice were transferred with CD45.1 WT BM cells.[22] Two months later, the reconstituted mice were used.

Statistical Analyses. All data are shown as the mean ± standard deviation of samples in each experimental group. Five to seven mice were used for each experimental group. Significance between the experimental and control groups was examined by the unpaired Student *t* test. *P* values less than 0.05 were considered significant. Two to three experiments were

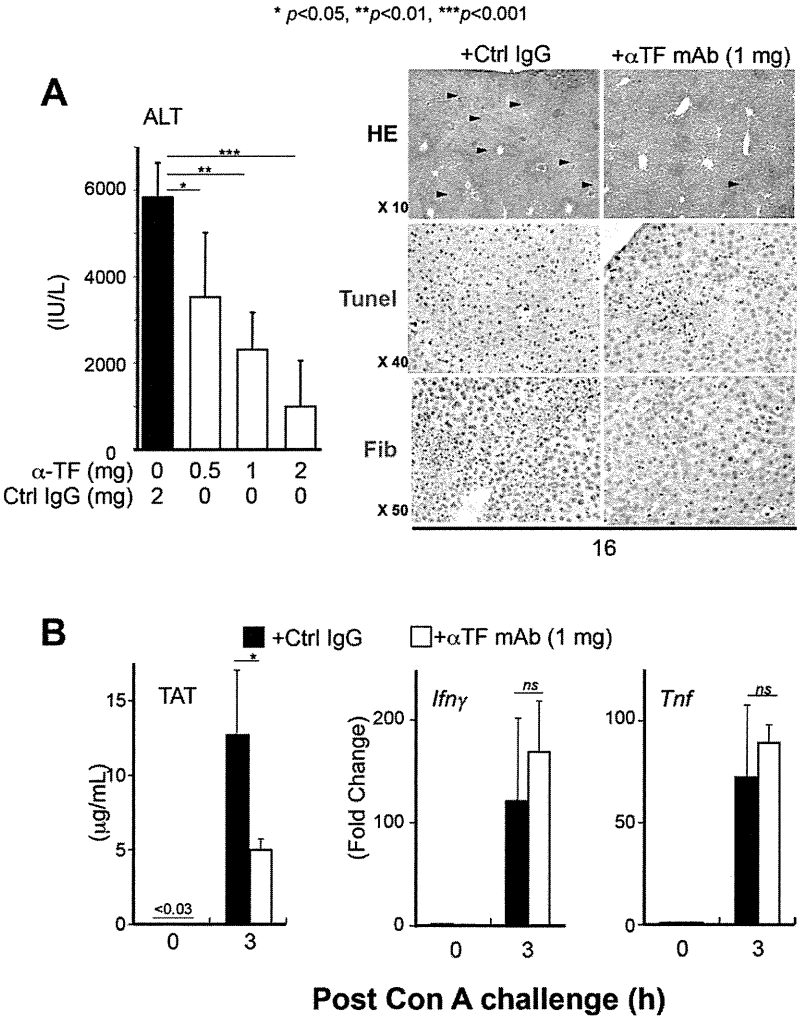

Fig. 3. TF is necessary for the development of Con A hepatitis. We administered various doses of neutralizing anti-TF mAb (open bars) or control rat IgG (Ctrl IgG) (closed bars) into WT mice 30 minutes before Con A challenge. At the indicated time points after Con A challenge, plasma and liver specimens were sampled for measurement of ALT (A) and TAT (B) as well as measurement of *Ifnγ* and *Tnf* expressions (B) and histological/immunohistological studies (A), respectively. Arrowheads indicated necrotic area.

separately performed, and representative data were shown.

Results

IFN-γ- and TNF-Dependent Hepatic Hypercoagulation Underlies Con A–Induced Hepatitis. We previously reported that by use of electron microscopy, many microthrombi, consisting of platelets, red blood cells, and fibrin deposits were observed in hepatic sinusoids of Con A–treated mice.[2] Immunohistochemistry (IHC) with antifibrinogen Abs further substantiated the dense fibrin deposition in the hepatic sinusoids (Fig. 1A, right middle lower panel). Pretreatment with the anticoagulant, heparin, protected against Con A–induced liver injuries with abundant TUNEL-positive hepatocytes and resulted in greatly reduced fibrin deposition (Fig. 1A). Consistent with our previous report,[2] heparin pretreatment did not down-regulate hepatic *Ifnγ* or *Tnf* (Fig. 1B). This is also true for interleukin-1β (*Il1β*), interleukin-6 (*Il6*), and CC chemokine ligand 2 (*Ccl2*) genes (Supporting Fig. 1). These results clearly indicated the importance of intrahepatic fibrin deposition for liver injury, and suggested that induction of these proinflammatory cytokines/chemokine was insufficient for the development of liver injuries in the absence of hepatic thrombosis.

Because plasma TAT is an excellent indicator of thrombin formation in the circulation,[16] we measured plasma TAT levels of Con A–challenged mice. Concomitant with dense fibrin deposition in the liver (Fig. 1A), plasma TAT levels were strongly elevated after challenge of WT mice with Con A, indicating that Con A treatment induced a systemic coagulation response along with hepatic hypercoagulation. Our previous report revealed that blockade of IFN-γ and

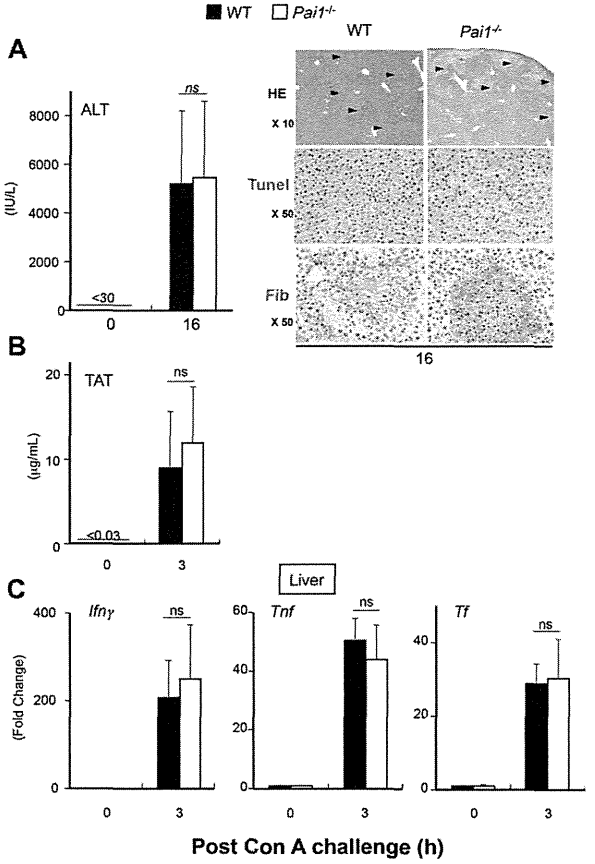

Fig. 4. Dispensability of PAI-1. WT mice (closed bars) or *Pai1*$^{-/-}$ mice (open bars) were challenged with Con A. At the indicated time points after Con A challenge, plasma and liver specimens were sampled, followed by the experiments, as shown in the legend to Fig. 3. Arrowheads indicated necrotic area.

TNF reduced hepatic coagulation response.[2] The involvement of IFN-γ and TNF was examined in more detail by using knockout (KO) mice, including single- and double-KO mice. Expectedly, *Ifnγ*$^{-/-}$, *Tnf*$^{-/-}$, and *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice had lower TAT elevation than WT mice (Fig. 1C). This clearly indicated the requirement of IFN-γ and TNF for the Con A–induced hypercoagulation response. In agreement, *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice lacked fibrin deposition and were protected from Con A–induced hepatitis (Fig. 1D). *Ifnγ*$^{-/-}$ mice, like *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice, were free from liver injury, whereas *Tnf*$^{-/-}$ mice showed only partial reduction of liver injury (Fig. 1D, right panel), suggesting that endogenous IFN-γ is more important than TNF for promoting liver injury. In contrast, *Ifnγ*$^{-/-}$ *Tnf*$^{-/-}$ mice showed significantly reduced, but still substantial induction of, *Il1β*, *Il6*, and *Ccl2* (Supporting Fig. 2). Taken together, these results demonstrated that both IFN-γ and TNF are important initiators in the development of massive liver necrosis, which is mediated by the induction of intrahepatic hypercoagulation.

***Requirement of IFN-γ and TNF for Hepatic Induction of* Tf *and* Pai1.** Next, we investigated how IFN-γ and/or TNF contributed to hepatic thrombosis. Because TF and PAI-1 were reported to induce the prothrombotic state,[5,9] we measured both *Tf* and *Pai1* levels in livers of Con A–challenged WT mice. Hepatic *Tf* levels started to increase at 2 hours, with a peak at 3 hours after Con A challenge (Fig. 2A,B). Hepatic *Pai1* levels began to increase at 1 hour and peaked at approximately 3-6 hours (Fig. 2A,B). Intriguingly, both *Ifnγ* and *Tnf* levels increased immediately after Con A challenge, and the peaks of *Ifnγ* and *Tnf* preceded those of *Tf* and *Pai1* (Fig. 2A). In sharp contrast to WT mice, *Ifnγ*$^{-/-}$ mice showed no increase in *Tf* and only little increase in *Pai1* levels (Fig. 2B), indicating the importance of IFN-γ for the induction of both *Tf* and *Pai1*. This was also the case for *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice (Fig. 2B). *Tnf*$^{-/-}$ mice showed poor induction of *Tf* and *Pai1* as well, but their levels were significantly higher than those of *Ifnγ*$^{-/-}$ mice and *Ifnγ*$^{-/-}$ *Tnf*$^{-/-}$ mice (Fig. 2B). Compared to *Tf* and *Pai1*, the Con A–mediated increase of messenger RNA (mRNA) levels of tPA, a target protease of PAI-1, were much less pronounced and peaked at a much later time point in WT mice (Fig. 2B). In addition, *tpa* levels were only slightly reduced in *Ifnγ*$^{-/-}$, *Tnf*$^{-/-}$, and *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice (Fig. 2B). These results strongly suggested that Con A stimulates hepatic T cells to produce both IFN-γ and TNF, which then induce the expression of hepatic *Tf* and *Pai1*.

Importance of TF, but Not PAI-1, for Liver Injuries. To examine the respective roles of TF and PAI-1 for the hypercoagulation response, we determined the effects of Con A treatment in WT mice pretreated with an anti-TF mAb and in *Pai1*$^{-/-}$ mice. Compared to mice receiving control rat IgG, treatment with the neutralizing anti-TF mAb, 1H1, just before Con A challenge reduced ALT plasma levels and fibrin deposition in a concentration-dependent manner (Fig. 3A). This indicated the importance of TF in mediating liver injury. Notably, TF blockade protected against plasma elevation of TAT without affecting hepatic *Ifnγ*, *Tnf*, *Il1β*, *Il6*, and *Ccl2* inductions (Fig. 3B and Supporting Fig. 3). In contrast, *Pai1*$^{-/-}$ mice underwent massive liver injuries similar to WT mice in respect to hepatic fibrin deposition, plasma TAT elevation and induction of hepatic *Ifnγ*, *Tnf*, and *Tf* (Fig. 4). These results demonstrated a pivotal role for TF, but not PAI-1, in hypercoagulation response and the development of liver injuries.

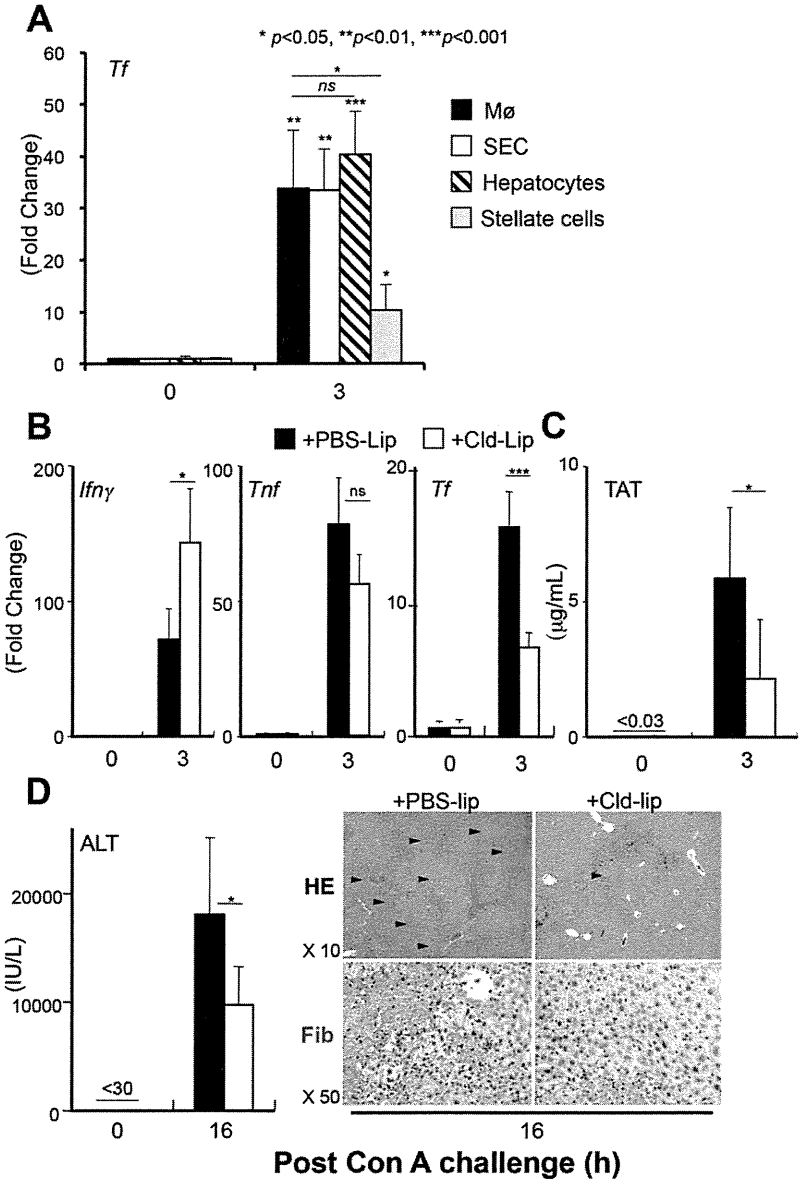

Fig. 5. Importance of macrophages for hepatic fibrin deposition. (A) Hepatic Mø (closed bars), SECs (open bars), hepatocytes (hatched bars), and stellate cells (gray bars) were isolated from WT mice at the indicated time points after Con A challenge. *Tf* was measured by qRT-PCR (A). (B-D) WT mice, having received clodronate liposome (Cld-lip) or control PBS liposome (PBS-lip), were challenged with Con A. At the indicated time points after Con A challenge, plasma and liver specimens were sampled, followed by the method shown in the legend to Fig. 1. Arrowheads indicated necrotic area.

***Liver Cells Both Inside and Outside of the Sinusoid Expressed* Tf.** Various cell types are localized within the hepatic sinusoid, such as SECs and liver Mø, including Kupffer cells. To identify the cell types that expressed TF mRNA upon Con A challenge, we isolated hepatic $CD11b^+$ Mø and $CD146^+$ SECs from Con A–treated mice and measured *Tf* expression. Both Mø and SECs prepared from livers of mice at 3 hours after Con A challenge showed a remarkable increase in *Tf* expression levels, as compared to naïve mice (Fig. 5A). Furthermore, cells outside of the sinusoid, such as hepatocytes and stellate cells, also increased the expression of *Tf* after Con A challenge (Fig. 5A). These results suggested that TF on Mø and SECs directly triggered the coagulation cascade within the hepatic sinusoid.

To analyze the roles of Mø in liver thrombosis, we generated Mø-depleted mice by injection of clodronate liposome.[15] Upon Con A challenge, Mø-depleted WT mice displayed significant diminution in hepatic *Tf* induction without reduction in hepatic *Ifnγ* and *Tnf* induction, as compared to PBS liposome-pretreated control mice (Fig. 5B). This suggested that the impaired TF induction was not attributed to the impaired induction of the upstream cytokines, but was rather the result of a decrease in the number of

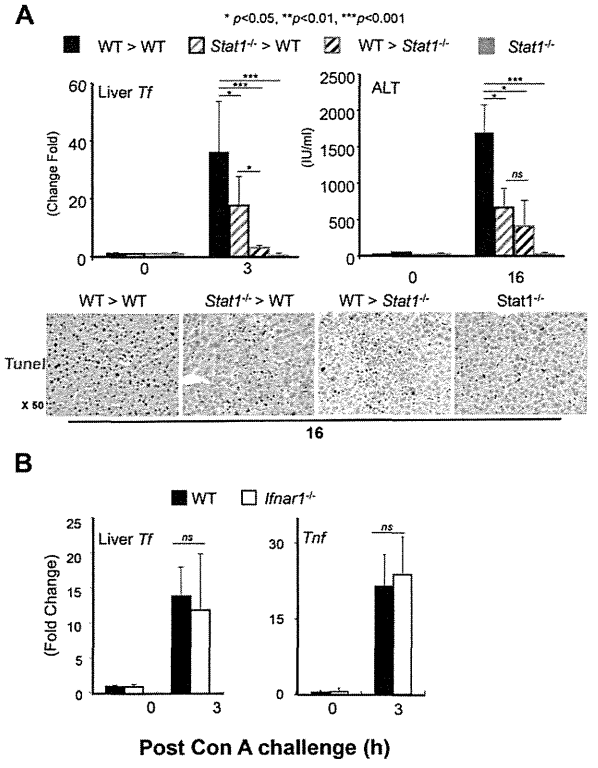

Fig. 6. A pivotal role of IFN-γ/STAT1 signaling in both hematopoietic and nonhematopoietic cells in Con A hepatitis. (A) Host mice were treated with clodronate liposome 2 days before reconstitution. After irradiation, host CD45.1 B6 WT received BM cells from congenic B6 CD45.2 WT (WT>WT) or *Stat1*$^{-/-}$ CD45.2 B6 mice (*Stat1*$^{-/-}$>WT). Irradiated *Stat1*$^{-/-}$ B6 CD45.2 mice received BM cells from CD45.1 B6 WT (WT>*Stat1*$^{-/-}$). *Stat1*$^{-/-}$ mice were used as well. The reconstituted mice were challenged with Con A. At the indicated time points, plasma and liver specimens were sampled for measurement of hepatic *Tf*, TUNEL assay, and plasma ALT levels. (B) WT mice (closed bars) or *Ifnar1*$^{-/-}$ mice (open bars) were challenged with Con A. *ns*, not significant.

TF-expressing cells. Furthermore, Mø-depleted mice showed impairment in plasma TAT increase, hepatic fibrin deposition, and liver injuries (Fig. 5C,D). The findings suggested that Mø were an important cellular source of functional TF.

Requirement of IFN-γ/STAT1 Signaling in Both Mø and SECs for the Hepatic* Tf *Induction. Because endogenous IFN-γ appeared more important than TNF for hepatic *Tf* induction (Fig. 2B), we further investigated the IFN-γ signaling pathway in liver cells (Fig. 6A). *Stat1*$^{-/-}$ mice, like *Ifnγ*$^{-/-}$ mice (Fig. 1D), showed a strongly impaired hepatic *Tf* induction and completely evaded Con A hepatitis (Fig. 6A), indicating the importance of the IFN-γ/STAT1-signaling pathway for these events. To exclude the possible involvement of type I IFN-mediated STAT1 signaling, we carried out experiments with mice deficient in the receptor for type I IFN, IFNAR. *Ifnar*$^{-/-}$ mice displayed healthy hepatic induction of *Tf* and *Tnf* (Fig. 6B), indicating that STAT1-mediated *Tf* up-regulation is not dependent on type I IFN. Next, we examined whether hepatic Mø or nonhematopoietic liver cells, including SECs, hepatocytes, and stellate cells, were responsible for STAT1-dependent *Tf* expression. We generated reciprocal BM chimeric mice by using WT and *Stat1*$^{-/-}$ mice. Mø are somewhat irradiation resistant. To improve depletion of host Mø, we pretreated host mice with clodronate liposome before reconstitution.[22] WT mice reconstituted with WT hematopoietic cells (control mice) showed *Tf* induction in their livers after Con A challenge (Fig. 6A). WT mice transferred with *Stat1*$^{-/-}$ BM cells exhibited partly impaired induction of *Tf*, as compared to the control mice (Fig. 6A). *Stat1*$^{-/-}$ mice reconstituted with WT hematopoietic cells showed further reduction in *Tf* induction, as compared to *Stat1*$^{-/-}$ mice receiving WT BM cells (Fig. 6A). IHC with antiphosphorylated STAT1 mAb revealed its nuclear localization in the corresponding WT Mø and nonhematopoietic liver cells of the chimeric mice (Supporting Fig. 4). Thus, the *Tf* inductions in Mø and nonhematopoietic liver cells were largely dependent on STAT1. WT mice transferred with *Stat1*$^{-/-}$ hematopoietic cells and *Stat1*$^{-/-}$ mice with WT BM cells both developed significantly mild liver injuries, compared to control mice (Fig. 6A). Intriguingly, severities of the liver injuries were comparable between these two types of chimeric mice (Fig. 6A). *Stat1*$^{-/-}$ mice reconstituted with *Stat1*$^{-/-}$ BM cells exhibited the phenotypes equivalent to *Stat1*$^{-/-}$ mice (data not shown). Collectively, these results strongly indicated that the IFN-γ/STAT1 signalings in both hematopoietic Mø and nonhematopoietic liver cells are equally important for the development of Con A hepatitis.

Con A Signaling in Non-T Non-B Cells Collaborates With IFN-γ and TNF Signaling in Thrombosis-Mediated Liver Injury. T cells have been documented to be essential for Con A hepatitis.[1] In agreement, *Rag2*$^{-/-}$ mice lacking T and B cells did not show hepatic *Tf* induction, elevation of plasma TAT concentrations, or liver damage after Con A challenge (Fig. 7A). T cells, including natural killer T cells, are necessary for the production of IFN-γ and TNF.[2,23] Both *Ifnγ* and *Tnf* inductions were absent in the liver of Con A–challenged *Rag2*$^{-/-}$ mice (Supporting Fig. 5). Because both IFN-γ and TNF mediate hypercoagulation and liver injury (Fig. 1C,D), we hypothesized that T cells may contribute to liver damage by producing IFN-γ and TNF. To test this possibility, we

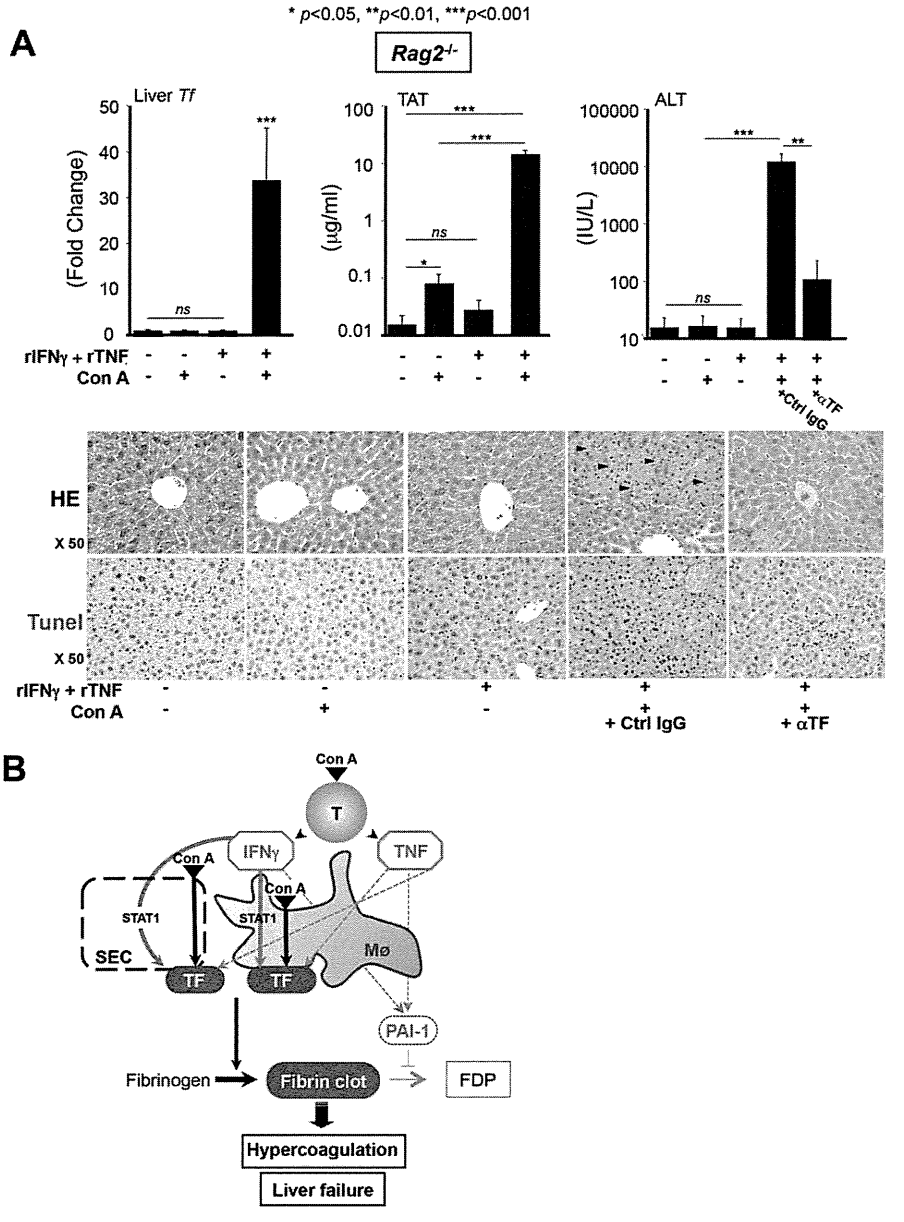

Fig. 7. IFNγ, TNF, and Con A signalings, likely in hepatic Mø and SECs, are necessary and sufficient for hepatitis involving hypercoagulation. (A) *Rag2*$^{-/-}$ mice were treated with rIFN-γ plus rTNF, Con A or Con A plus rIFNγ plus rTNF, or, additionally, with control rat IgG (Ctrl IgG) or neutralizing anti-TF mAb (αTF). At 3 hours, plasma and liver specimens were sampled for measurement of TAT and *Tf* expression, respectively. At 9 hours, plasma and liver specimens were sampled for measurement of ALT and histological study, respectively. *ns*, not significant. Arrowheads indicated necrotic area. (B) A *propo*sal model for Con A–induced liver injury. Upon Con A challenge, T cells produce IFN-γ and TNF, which, together with Con A, cooperatively stimulate sinusoidal Mø and SECs to produce TF in a STAT1-dependent manner. TF then starts to rapidly and aberrantly activate the coagulation cascade to generate hepatic sinusoidal thrombus, eventually leading to massive liver injuries.

administered rIFN-γ plus rTNF into *Rag2*$^{-/-}$ mice and challenged them with Con A. This treatment resulted in hepatitis accompanied by considerable hepatic *Tf* induction, along with *Il1β*, *Il6*, and *Ccl2* inductions (Supporting Fig. 6) and a strong increase in plasma TAT levels (Fig. 7A). Notably, TF blockade protected against the elevation of plasma TAT levels and the liver injuries in Con A plus rIFN-γ/rTNF-treated *Rag2*$^{-/-}$ mice (Fig. 7A and Supporting Fig. 7). However, in the absence of Con A, treatment with IFN-γ and TNF alone could not induce any of those alterations (Fig. 7A). Thus, in addition to signals elicited by IFN-γ and TNF, Con A signaling in the cells of *Rag2*$^{-/-}$ mice, likely mediated by hepatic Mø and SECs, was required for the development of thrombosis-mediated liver injuries.

Discussion

Results presented here demonstrate that both endogenous IFN-γ and TNF are essential for the development of Con A–induced liver injuries through the induction of TF-dependent coagulation. In particular, the IFN-γ/STAT1-signaling pathway, in both hepatic

Mø and SECs, was directly critical for the development of hypercoagulation and resultant acute liver injuries (ALIs). However, exogenous or endogenous IFN-γ and TNF were not sufficient to induce the hypercoagulation response or liver injuries. However, exogenous IFN-γ and TNF rendered *Rag2*$^{-/-}$ mice highly susceptible to Con A treatment, suggesting that Con A, IFN-γ, and TNF act in concert on hepatic Mø and SECs to elicit a procoagulant response. Based on these results, we propose a model of Con A–induced acute liver damage (illustrated in Fig. 7B). After stimulation with Con A, T cells produce IFN-γ and TNF. In hepatic Mø and SECs within the sinusoid, the cellular signaling pathways initiated by Con A, TNF, and IFN-γ through STAT1 activation synergize to elicit a robust expression of TF. TF then activates the coagulation system, leading to hepatic fibrin deposition and liver injury.

The IFN-γ/STAT1-mediated signaling in SECs is important for Con A–induced liver injury. Mø-depleted *Stat1*$^{-/-}$ mice reconstituted with WT Mø showed reduction in hepatic *Tf* induction and evaded Con A–induced liver injury. This suggested that IFN-γ/STAT1 induction of TF in SECs might evoke the hypercoagulation response relevant to the liver injury. A recent report verified a crucial role of endogenous IFN-γ in SEC damage of Con A–treated mice.[24] SEC damage has been believed to be a potent inducer of intrahepatic coagulation.[25] Therefore, IFN-γ/STAT1-mediated induction of TF in SECs may contribute to intrahepatic coagulation within the context of IFN-γ/STAT1-mediated cellular damage.

Under normal conditions, hepatocytes and stellate cells are anatomically segregated from the sinusoid. However, IFN-γ induction of SEC damage allows them to be exposed to the sinusoidal circulation, which might facilitate thrombosis. Thus, IFN-γ/STAT1-mediated induction of TF in hepatocytes and stellate cells might amplify procoagulant response.

Con A is a well-known T-cell mitogen, suggesting an important role of T cells in Con A–induced hypercoagulation. However, our present results verified the replacement of T cells by IFN-γ/TNF and the importance of Con A signaling in non-T cells, presumably exemplified by hepatic Mø and SECs. We are currently investigating the signaling pathway of Con A in Mø and SECs.

Thrombin-cleaved osteopontin was shown to be involved in this type of hepatitis.[23,26] These reports are consistent with the view that hepatic thrombosis is an essential contributor to Con A–induced liver injuries, at least through the induction of the thrombin-cleaved form of osteopontin and, perhaps, hepatic microcirculatory disturbance.

Mouse hepatitis virus infection is associated with intrahepatic thrombosis.[27,28] Patients with chronic hepatitis C show increase in plasma TF levels, whereas those with viral clearance by IFN-α therapy, such as healthy controls, do not.[29] Furthermore, there is a growing recognition of the role of hypercoagulation in chronic liver injury and fibrosis.[30] Although our current study was focused on ALI, the similar mechanism likely underlies acute and chronic viral hepatitis and fibrosis.

In summary, our present study demonstrates that IFN-γ/STAT1-mediated signaling in hepatic various cells, including Mø and SECs, is the underlying mechanism of Con A–induced aberrant activation of coagulation resulting in massive liver necrosis. In hepatic Mø and liver sinusoid, signaling through the IFN-γ/STAT1 pathway induced expression of *Tf*, which, in conjunction with IFN-γ/STAT1-mediated damage to the endothelium, triggered the coagulation reactions. The resulting formation of extensive microthrombi induced microcirculatory disturbances and hepatic inflammation involving thrombin-mediated conversion of precursor proteins, eventually leading to massive liver necrosis. It is conceivable that similar mechanisms are driving the progression of lethal fulminant hepatitis. Although our study did not formally address this question, the findings presented here may incite future studies to investigate this possibility and, perhaps, lead to novel therapeutic approaches.

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Original Article

Survey of non-B, non-C liver cirrhosis in Japan

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Aim: The aim of this survey was to reveal clinical features for each etiology of non-B, non-C liver cirrhosis (NBNC LC) in Japan.

Methods: In a nationwide survey of NBNC LC in Japan at the 15th General Meeting of the Japan Society of Hepatology, 6999 NBNC LC patients were registered at 48 medical institutions. Epidemiological and clinical factors were investigated.

Results: The percentage of NBNC LC among LC patients was 26%. NBNC LC patients were categorized into 11 types according to etiological agents: non-alcoholic steatohepatitis (NASH), 14.5%; alcoholic liver disease (ALD), 55.1%; fatty liver disease (FLD), except NASH, ALD, and other known etiology, 2.5%; primary biliary cirrhosis, 8.0%; other biliary cirrhosis, 0.8%; autoimmune hepatitis, 6.8%; metabolic disease, 0.6%; congestive disease, 0.8%; parasitic disease, 0.2%; other known etiology, 0.2%; and unknown etiology, 10.5%. Compared with previous surveys, the percentage of ALD remained unchanged, whereas that of NASH increased. The mean age and percentage of females were significantly higher in NASH patients than in ALD and FLD patients. Prevalence of diabetes mellitus was significantly higher in NASH and FLD patients than in ALD ones. Prevalence of hepatocellular carcinoma (HCC) in NBNC LC patients was 35.9%. Among NASH, ALD and FLD patients, 50.9%, 34.3% and 54.5% had HCC, respectively. Positivity of hepatitis B core antibody was significantly higher in HCC patients than in those without HCC (41.1% vs 24.8%).

Conclusion: This survey determined the etiology of NBNC LC in Japan. These results should contribute new ideas toward understanding NBNC LC and NBNC HCC.

Key words: alcoholic liver disease, hepatocellular carcinoma, non-alcoholic steatohepatitis, non-B, non-C liver cirrhosis

INTRODUCTION

A NATIONWIDE SURVEY of liver cirrhosis (LC) for each etiology has been conducted as the main theme on four occasions at the national academic conference in Japan. Therefore, many registered patients have been surveyed on uniform diagnostic criteria.[1] The 15th General Meeting of the Japan Society of Hepatology was held in October 2011. In a featured session in this meeting, we conducted a nationwide survey of non-B, non-C LC (NBNC LC) in patients at medical institutions in Japan. NBNC LC was the main theme of the featured session in this meeting for two reasons. First, the prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing and has been recently reported to be approximately 20% in adults in Japan. Approximately 1% of adults in Japan are estimated to have non-alcoholic steatohepatitis (NASH).[2,3] Thus, NASH is the most common chronic liver disease not only in Western countries but also in Japan. NASH patients can develop LC and even hepatocellular carcinoma (HCC), although there have been few investigations concerning the incidence of LC associated with NASH (NASH LC)

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in Japan. Second, the number of NBNC HCC patients has been rapidly increasing, and it has been recently reported to account for approximately 15% of all HCC patients in Japan.[4] Most NBNC HCC patients seem to have LC with alcoholic liver disease (ALD LC); however, NASH LC has been noted as a high-risk group of NBNC HCC. Nevertheless, HCC complicated with NBNC LC of an unknown cause has been occasionally reported. Therefore, it is important to investigate the clinical features of NBNC LC, which will lead to the development NBNC HCC. Based on these backgrounds, we report the characteristics of NBNC LC in Japan. This was one of the programs of the 15th General Meeting of the Japan Society of Hepatology in 2011.

METHODS

Patient database

AT 48 MEDICAL institutions (all investigators are listed in Appendix I) (Table 1), 6999 subjects were diagnosed with NBNC LC based on the negative results for serum hepatitis B surface antigen (HBsAg), anti-hepatitis C antibody and hepatitis C virus (HCV) RNA. The patients registered in this study were clinically (laboratory examinations and imaging studies) and histologically diagnosed with LC based on the criteria proposed by a previous nationwide survey (the 44th Annual Meeting of the Japan Society of Hepatology in 2008).[1] The NBNC LC patients were categorized into 11 types according to etiology: (i) NASH; (ii) ALD; (iii) fatty liver disease (FLD); (iv) primary biliary cirrhosis (PBC); (v) other biliary cirrhosis (such as primary sclerosing cholangitis [PSC] and secondary biliary cirrhosis); (vi) autoimmune hepatitis (AIH) (including AIH-PBC overlap syndrome); (vii) metabolic disease (such as Wilson's disease, hemochromatosis and glycogen storage disease); (viii) congestive disease (including Budd–Chiari syndrome); (ix) parasitic disease (such as Japanese schistosomiasis); (x) other known etiology (such as sarcoidosis and drug-induced liver injury); and (xi) unknown etiology. The diagnosis of NASH was based on the following criteria: (i) absence of clinically significant alcohol consumption (intake of ≤20 g ethanol/day); (ii) appropriate exclusion of other liver diseases; (iii) complications with risk factors of steatosis such as obesity (in particular, visceral obesity), metabolic syndrome and diabetes mellitus; and (iv) the presence of steatosis on liver histology (histological

Table 1 Forty-eight medical institutions registered at the 15th General Meeting of the Japan Society of Hepatology on 2011

Akita University Graduate School of Medicine	Nara Medical University
Asahikawa-Kosei General Hospital	National Center for Global Health and Medicine
Asahikawa Medical University	Nihon University School of Medicine
(Division of Gastroenterology and Hematology/Oncology)	Niigata Prefectural Central Hospital
(Division of Metabolism and Biosystemic Science)	Niigata University Medical and Dental Hospital
Asahikawa Red Cross Hospital	Oji General Hospital
Chiba University	Osaka City University
Dokkyo Medical University	Osaka Police Hospital
Ehime Prefectural Central Hospital	Osaka Red Cross Hospital
Ehime University Graduate School of Medicine	Saiseikai Suita Hospital
Fukushima Medical University School of Medicine	Saitama Medical University
Gunma University Graduate School of Medicine	Sapporo City General Hospital
Hyogo College of Medicine	Sapporo-Kosei General Hospital
Iwate Medical University	Shinshu University School of Medicine
Jikei University School of Medicine, Katsushika Medical Center	Teikyo University School of Medicine
Juntendo University School of Medicine	Teine-Keijinkai Hospital
Kagawa University	Tokyo Medical and Dental University
Kanazawa Medical University	Tokyo Medical University Ibaraki Medical Center
Keio University School of Medicine	Tokyo Women's Medical University
Kumamoto University	Tottori University School of Medicine
Kurume University School of Medicine	University of Tokyo
Kyoto Second Red Cross Hospital	University of Yamanashi
Mie University Graduate School of Medicine	(First Department of Internal Medicine)
Musashino Red Cross Hospital	(First Department of Surgery)
Nagano Red Cross Hospital	Yamagata University Faculty of Medicine

diagnosis) or imaging studies (imaging diagnosis). The diagnosis of ALD was based on the proposed Diagnostic Criteria for Alcoholic Liver Disease by a Japanese study group for ALD (the Takada group).[5] The diagnosis of FLD was based on the following criteria: (i) alcohol consumption between that for NASH and ALD (i.e. intake of >20 g and <70 g ethanol/day); (ii) appropriate exclusion of other liver diseases; and (iii) the presence of steatosis on liver histology or imaging studies.

The following variables were used to investigate the clinical features of NBNC LC: age; sex; body mass index (BMI); prevalence of diabetes mellitus (DM), impaired glucose tolerance, hypertension and dyslipidemia; Child–Pugh classification; prevalence of gastroesophageal varices and HCC; and presence of hepatitis B core antibody (anti-HBc). In addition, the percentage of NBNC LC was investigated among all LC patients at each institution and was compared with previous reports. The ethics committees of the appropriate institutional review boards approved this study in accordance with the Declaration of Helsinki (2000).

Statistical analyses

Statistical tests were performed using the IBM SPSS Statistics ver. 21. The statistical significance of difference was determined using the χ^2-test, Mann–Whitney *U*-test and multivariate Cox's proportional hazard model as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Percentage of NBNC LC among all LC patients

WE CALCULATED THE percentage of NBNC LC among all 25 020 LC patients at 37 registered institutions. The percentages of NBNC LC, hepatitis B virus (HBV)-related cirrhosis, HCV-related cirrhosis, and both HBV- and HCV-related cirrhosis were 26%, 12%, 60.9% and 1.1%, respectively. Compared with a previous nationwide survey (the 44th Annual Meeting of the Japan Society of Hepatology in 2008),[1] there was no significant difference between them (Table 2).

Frequency of each etiology among NBNC LC patients

We determined the frequency and percentage of each etiology among all 6999 NBNC LC patients at 48 registered institutions. The percentages of each etiology were as follows: NASH, 14.5%; ALD, 55.1%; FLD, 2.5%; PBC, 8.0%; other biliary cirrhosis, 0.8%; AIH, 6.8%; metabolic disease, 0.6%; congestive disease, 0.8%; parasitic disease, 0.2%; other known etiology, 0.2%; and unknown etiology, 10.5% (Table 3). Among 1015 NASH patients, 309 (30.4%) were diagnosed histologically, 402 (39.6%) were diagnosed by imaging studies and the method of diagnosis of 304 patients (30%) was not described in detail. Among 60 patients with other biliary cirrhosis, 71.7% had PSC and the rest had cholestatic diseases, except PBC and PSC (such as congenital biliary atresia and secondary biliary cirrhosis). Among 39 metabolic disease patients, 66.7% had Wilson's disease, 25.6% had hemochromatosis (glycogen storage disease, amyloidosis and citrullinemia in one patient each). All 12 cases of parasitic disease were Japanese schistosomiasis. Of 11 patients with other known etiology, two patients sarcoidosis, two post-liver transplantation, two post-hepatectomy, one drug-induced liver injury, one systemic lupus erythematosus-related liver injury and the diagnosis of the remaining patients was not described in detail.

Compared with the survey at the 44th Annual Meeting of the Japan Society of Hepatology in 2008,[1] the percentage of ALD among all NBNC LC patients did

Table 2 Percentage of NBNC LC among all patients with liver cirrhosis compared with the 44th Annual Meeting of the Japan Society of Hepatology on 2008[1]

	The 15th General Meeting of the Japan Society of Hepatology on 2011 ($n = 25\,020$)	The 44th Annual Meeting of the Japan Society of Hepatology on 2008 ($n = 33\,379$)	*P*-value
NBNC LC	26.0%	24.0%	N.S.
HBV-related cirrhosis	12.0%	13.9%	N.S.
HCV-related cirrhosis	60.9%	60.9%	N.S.
both HBV- and HCV-related cirrhosis	1.1%	1.2%	N.S.

P-values were analyzed by χ^2-test.
HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant.

Table 3 Frequency of each etiology among patients with NBNC LC compared with the 44th Annual Meeting of the Japan Society of Hepatology on 2008[1]

	The 15th General Meeting of the Japan Society of Hepatology on 2011 ($n = 6999$)	The 44th Annual Meeting of the Japan Society of Hepatology on 2008 ($n = 8011$)	*P*-value
NASH	14.5%	8.7%	$P < 0.001$
ALD	55.1%	56.3%	N.S.
FLD	2.5%	–	–
PBC	8.0%	9.9%	$P < 0.001$
Other biliary cirrhosis	0.8%	1.2%	$P < 0.001$
AIH	6.8%	7.9%	$P = 0.018$
Metabolic disease	0.6%	1.2%	$P < 0.001$
Congestive disease	0.8%	1.2%	$P = 0.013$
Parasites	0.2%	0.4%	$P = 0.011$
Other known etiology	0.2%	0.8%	$P < 0.001$
Unknown etiology	10.5%	12.4%	$P < 0.001$

P-values were analyzed by χ^2-test.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant; PBC, primary biliary cirrhosis.

not change (55.1% vs 56.3%), whereas that of NASH increased (14.5% vs 8.7%; $P < 0.001$) (Table 3).

Clinical features of NBNC LC patients

The male : female ratio for the NBNC LC patients was 1.93. The percentages of each etiology among 4608 male and 2391 female patients were as follows: NASH (9.5% and 24%), ALD (73.4% and 19.8%), FLD (3.4% and 0.9%), PBC (1.9% and 20%), other biliary cirrhosis (0.8% and 0.9%), AIH (1.5% and 17.1%), metabolic disease (0.5% and 0.8%), congestive disease (0.8% and 0.8%), parasitic disease (0.2% and 0.1%), other known etiology (0.1% and 0.2%) and unknown etiology (7.9% and 15.4%), respectively (Fig. 1). The male : female ratio for each etiology among the NBNC LC patients was as follows: NASH, 0.77; ALD, 7.12; FLD, 6.86; PBC, 0.18; other biliary cirrhosis, 1.73; AIH, 0.17; metabolic disease, 1.29; congestive disease, 2.17; parasitic disease, 5; other known etiology, 0.83; and unknown etiology, 0.99 (Table 4). Thus, the NASH patients were predominantly female as opposed to the ALD and FLD patients who were predominantly male.

The mean age at clinical diagnosis in the NBNC LC patients for NASH, ALD, FLD, PBC, other biliary cirrhosis, AIH, metabolic disease, congestive disease, parasitic disease, other known etiology and unknown etiology was 66.9, 60.3, 64.2, 63.6, 51.3, 64.5, 42.6, 52.7, 77.4, 56.1 and 68.8 years, respectively. In the patients with NASH, AIH, congestive disease and unknown etiology, the mean ages at clinical diagnosis of the male patients were lower than those of the female patients ($P < 0.001$). In contrast, in the ALD, FLD, PBC and metabolic disease patients, the mean ages at clinical diagnosis of the female patients were lower than those of the male patients ($P < 0.001$) (Table 5).

Regarding the risk factors of NASH, the following variables were investigated in the NASH, ALD and FLD patients: BMI and the prevalence of DM, impaired glucose tolerance (IGT), hypertension and dyslipidemia. BMI in the NASH, ALD and FLD patients was 27, 23.4 and 25 kg/m^2, respectively, and the differences among them were statistically significant. The prevalence of DM and IGT in the NASH and FLD patients (63% and 57%, respectively) was significantly higher compared with that in the ALD patients (31%) ($P < 0.001$). The prevalence of dyslipidemia in the NASH and FLD patients (25% and 29%, respectively) was significantly higher compared with that in the ALD patients (14%) ($P < 0.001$). The prevalence of hypertension in the NASH patients (52%) was significantly higher compared with that in the ALD and FLD patients (28% and 35%, respectively) ($P < 0.001$) (Table 6).

The levels of hepatic functional reserve based on the Child–Pugh classification for each etiology are summarized in Table 7. The percentages of moderate-to-low hepatic reserve (Child–Pugh class B and C) in the ALD and AIH patients (52.9% in both) were significantly higher compared with those in the NASH and FLD patients (35.8% and 27%, respectively) ($P < 0.001$).

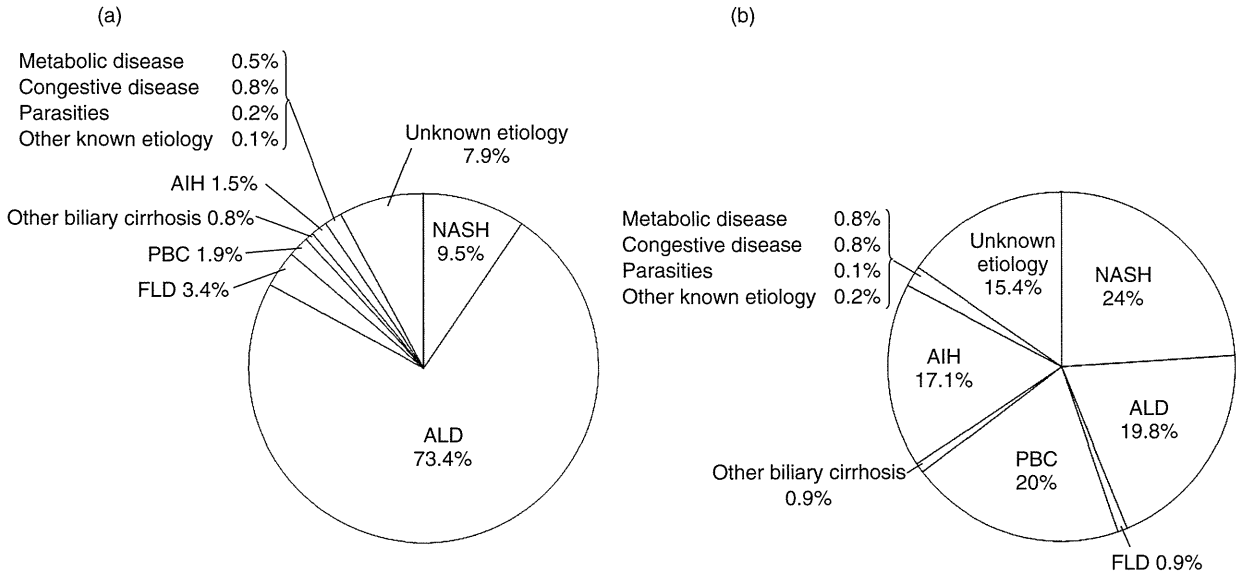

Figure 1 Frequency of each etiology among male or female patients with NBNC LC. (a) Male, (b) female. AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

To determine the frequency of complicated portal hypertension patients, the prevalence of gastroesophageal varices was calculated. The prevalence in the ALD and PBC patients (54.5% and 61.9%, respectively) was significantly higher compared with that in the patients with NASH, FLD, AIH and unknown etiology (40.8%, 40.7%, 48.2% and 45.9%, respectively) ($P < 0.05$). Considering only patients with Child–Pugh class A, the prevalence of gastroesophageal varices in PBC patients was highest among all etiologies. ALD had significantly higher prevalence than NASH, the histology of which was very similar (Table 8).

Table 4 Male : female ratio of each etiology

	Male ($n = 4608$)	Female ($n = 2391$)	Male : female ratio
NASH	440	575	0.77
ALD	3381	475	7.12
FLD	151	22	6.86
PBC	87	477	0.18
Other biliary cirrhosis	38	22	1.73
AIH	69	409	0.17
Metabolic disease	22	19	1.29
Congestive disease	39	18	2.17
Parasites	10	2	5.00
Other known etiology	5	4	0.83

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant; PBC, primary biliary cirrhosis.

The prevalence of HCC in the NBNC LC patients was 35.9%. Among 2438 NBNC HCC patients, 51.9% were diagnosed with HCC simultaneously with the diagnosis of NBNC LC, 25.6% were diagnosed after, 1.4% were diagnosed before the diagnosis of NBNC LC and the diagnosis of the remaining patients was not described in detail. The male : female ratio for the NBNC HCC patients was 3.06. The percentage of each etiology among the HCC patients was as follows: NASH, 19.9%; ALD, 53.4%; FLD, 3.7%; PBC, 3.2%; other biliary cirrhosis, 0.2%; AIH, 4.9%; metabolic disease, 0.1%; congestive disease, 0.7%; parasitic disease, 0.1%; other known etiology, 0%; and unknown etiology, 13.8%. The percentage of NASH among the NBNC HCC patients was significantly higher than that among the NBNC LC patients (19.9% vs 14.5%, $P < 0.001$). The clinical diagnosis of HCC was made at a mean age of 67.2 years in all patients. The mean age of onset of HCC was 70.8, 64.8 and 68.4 years in the NASH, ALD and FLD patients, respectively, and the differences among them were significant ($P < 0.001$). The prevalence of

Table 5 The mean ages at clinical diagnosis in the patients with NBNC LC

	Total ($n = 6999$)	Male ($n = 4608$)	Female ($n = 2391$)	P-value (M vs F)
NASH	66.9 ± 11.6	64.8 ± 13.2	68.5 ± 9.8	$P < 0.001$
ALD	60.3 ± 11.0	60.9 ± 10.7	55.7 ± 12.1	$P < 0.001$
FLD	64.2 ± 11.8	64.7 ± 11.3	61.2 ± 15.0	$P < 0.001$
PBC	63.6 ± 12.1	66.0 ± 11.3	63.2 ± 12.0	$P < 0.001$
Other biliary cirrhosis	51.3 ± 20.7	52.0 ± 22.0	50.0 ± 19.0	$P < 0.001$
AIH	64.5 ± 12.2	63.3 ± 14.2	66.0 ± 11.7	$P < 0.001$
Metabolic disease	42.6 ± 18.2	44.0 ± 18.0	40.7 ± 19.0	$P < 0.001$
Congestive disease	52.7 ± 20.4	50.5 ± 20.7	57.4 ± 19.6	$P < 0.001$
Parasites	77.4 ± 5.9	76.5 ± 6.1	81.5 ± 2.1	$P < 0.001$
Other known etiology	56.1 ± 19.1	53.0 ± 18.7	58.7 ± 20.8	$P < 0.001$
Unknown etiology	68.8 ± 11.9	67.9 ± 13.0	69.8 ± 10.7	$P < 0.001$

All results are expressed as mean ± standard deviation. P-values were analyzed by Mann–Whitney U-test.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

HCC in the patients with NASH, FLD and unknown etiology (50.9%, 54.5% and 47.5%, respectively) were significantly higher compared with that in the ALD, PBC and AIH patients (34.3%, 14.4% and 26.0%) ($P < 0.0001$). The percentage of moderate-to-low hepatic reserve (Child–Pugh class B and C) in HCC in AIH patients was significantly higher than those in the patients with NASH, FLD and unknown etiology

Table 6 Risk factors of NASH in the patients with NASH, ALD and FLD

Variable	NASH ($n = 1015$)	ALD ($n = 3856$)	FLD ($n = 173$)	P-value
Body mass index (kg/m^2)	27.0 ± 4.3	23.4 ± 6.4	25.0 ± 3.7	$P < 0.001$*,**
Diabetes mellitus or Impaired glucose tolerance	62.5%	37.5%	56.5%	$P < 0.001$*
Dyslipidemia	25.0%	13.5%	29.4%	$P < 0.001$* $P = 0.01$**
Hypertension	52.0%	28.2%	34.7%	$P < 0.001$*,**

ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis.
Results of body mass index are expressed as mean ± standard deviation. P-values were analyzed by Mann–Whitney U-test and χ^2-test.
*NASH vs ALD, **NASH vs FLD.

Table 7 Levels of hepatic functional reserve based on the Child–Pugh classification

Child–Pugh classification	Class A	Class B	Class C	Percentages of both class B and C	P-value
NASH ($n = 783$)	503	222	58	35.8%	
ALD ($n = 2710$)	1276	867	567	52.9%	$P < 0.001$*
FLD ($n = 89$)	65	18	6	27.0%	
PBC ($n = 355$)	204	105	46	42.5%	
AIH ($n = 295$)	139	106	50	52.9%	$P < 0.001$** $P = 0.01$***
Unknown etiology ($n = 515$)	300	150	65	41.7%	

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.
P-values were analyzed by χ^2-test.
*vs NASH, FLD, PBC and unknown etiology; **vs NASH and FLD; ***vs PBC and unknown etiology.

Table 8 Prevalence of patient with gastroesophageal varices

	Total	Child–Pugh classification		
		Class A	Class B	Class C
NASH ($n = 686$)	40.8% (280/686)*	31.8% (138/434)**	56.1% (111/198)	57.4% (31/54)
ALD ($n = 2365$)	54.5% (1289/2365)***	44.2% (486/1099)†	59.0% (447/757)	69.9% (356/509)
FLD ($n = 81$)	40.7% (33/81)	36.5% (23/63)	50.0% (7/14)	75.0% (3/4)
PBC ($n = 331$)	61.9% (205/331)††	53.5% (100/187)††	70.6% (72/102)	78.6% (33/42)
AIH ($n = 278$)	48.2% (134/278)	39.7% (52/131)	53.5% (53/99)	60.4% (29/48)
Unknown etiology ($n = 401$)	45.9% (184/401)	42.9% (94/219)	47.2% (60/127)	54.5% (30/55)

P-values were analyzed by Fisher's exact test or χ^2-test.
*$P < 0.05$, vs ALD, PBC and AIH; **$P < 0.01$, vs ALD, PBC and unknown etiology; ***$P < 0.05$, vs NASH, FLD and unknown etiology; †$P < 0.0001$ vs NASH; ††$P < 0.05$ vs NASH, ALD, FLD, AIH and unknown etiology.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis.

($P < 0.0001$). BMI in the NASH, ALD and FLD patients was 26.8, 24.0 and 25.8 kg/m^2, respectively, and the differences among them were statistically significant. (Table 9).

Table 10 shows the analysis of the risk factors associated with HCC in patients with ALD LC. Obesity and complication of DM were the risk factors of hepatic carcinogenesis in ALD LC patients as well as male sex and being older. Conversely, portal hypertension and anemia of ALD LC patients without HCC were worse than those with HCC. Accordingly, we investigated the comparison of the clinical features between the two ALD LC groups divided based on BMI (Table 11). Although the mean age was similar in these two groups, the prevalence of HCC in the ALD LC patients with obesity (BMI, ≥25 kg/m^2) was significantly higher compared with that in those without obesity (BMI, <25 kg/m^2) (48.3% vs 35.7%, $P < 0.001$) and similar to that in the NASH LC patients (48.3% vs 50.9%, not significant).

Of the NBNC LC patients, 31.3% were anti-HBc positive. Anti-HBc positivity was 30.7%, 30.8%, 34.7% and 43% in the patients with NASH, ALD, FLD and unknown etiology, respectively. The positivity was significantly higher in the patients with unknown etiology compared with the NASH, ALD and FLD patients ($P < 0.001$). Anti-HBc positivity was significantly higher in the HCC patients than in those without HCC (41.1% vs 24.8%, $P < 0.001$).

DISCUSSION

THIS NATIONWIDE SURVEY revealed the following clinical features in the NBNC LC patients:

1 Compared with the previous nationwide survey,[1] the percentage of ALD among the NBNC LC patients remained unchanged, whereas that of NASH increased.
2 The NASH LC patients were significantly older, predominantly female, heavier, hypertensive and more likely to have DM and HCC.
3 The ALD LC patients were significantly younger, predominantly male, had low hepatic reserve and were more likely to have portal hypertension than NASH LC.
4 The FLD LC patients were observed at an age between that of the NASH and ALD patients, were predominantly male (similar to the ALD patients) and were more likely to have DM and HCC similar to the NASH patients.
5 Approximately 10% of the NBNC LC patients still had an unknown etiology, and these patients were more likely to have HCC similar to both the NASH and FLD patients.
6 Anti-HBc positivity was significantly higher in the HCC patients than in those without HCC.

Although the natural history of NASH is not completely understood, Matteoni *et al.* reported that 23% of NASH patients progressed to cirrhosis within 10–15 years.[6] In addition, Starley *et al.* recently stated that approximately 26–37% of NASH patients demonstrate the progression of fibrosis over time periods up to 5.6 years, with up to 9% patients progressing to cirrhosis.[7] BMI and DM have been found to be independent risk factors associated with the progression of fibrosis in NASH patients.[8] Therefore, it is thought that the NASH LC patients in the present study had significantly more severe disease and were more likely to have DM. Conversely, the prevalence of NAFLD in Japan appears to be twice as high in males than in females;[9] however, the NASH LC patients in the present study were