Diabetes Accelerates Steatohepatitis in Mice

Liver Pathology and Single-Cell Gene Expression Signatures

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Glucose lowering independently reduces liver fibrosis in human nonalcoholic fatty liver disease. This study investigated the impact of diabetes on steatohepatitis and established a novel mouse model for diabetic steatohepatitis. Male C57BL/6J mice were fed a 60% high-fat diet (HFD) and injected with carbon tetrachloride (CCL4) and streptozotocin (STZ) to induce diabetes. The HFD+CCL4+STZ group showed more severe liver steatosis, hepatocyte ballooning, and regenerative nodules compared with other groups. Diabetes up-regulated inflammatory cytokine-associated genes and increased the M1/M2 macrophage ratios in the liver. Single-cell RNA sequencing analysis of nonparenchymal cells in the liver showed that diabetes reduced Kupffer cells and increased bone marrow–derived recruited inflammatory macrophages, such as Ly6Chi-RM. Diabetes globally reduced liver sinusoidal endothelial cells (LSECs). Furthermore, the up-regulation of genes related to the receptor for advanced glycation end products (RAGE)/Toll-like receptor 4 was observed in Ly6Chi-RM and LSECs in mice with diabetes. These findings open avenues for discovering novel therapeutic targets for diabetic steatohepatitis. (Am J Pathol 2024, 184: 1–15; https://doi.org/10.1016/j.ajpath.2024.01.007)

Nonalcoholic fatty liver disease (NAFLD) has developed into a worldwide health issue, progressing from simple steatosis to nonalcoholic steatohepatitis (NASH), eventually leading to liver fibrosis, liver failure, and hepatocellular carcinoma. Recognized risk factors for NASH include obesity and insulin resistance. Additionally, diabetes has been identified as a crucial risk factor for developing NASH-associated hepatocellular carcinoma. However, disentangling the impact of obesity and diabetes on NAFLD progression can be complex because these conditions often coexist, particularly in Western countries. Interestingly, in Asian populations, nonobese insulin secretory failure frequently is involved in the pathophysiology of type 2 diabetes.

In the clinical progression of NAFLD in Japanese subjects, an increase in hemoglobin A1c level, rather than weight gain, has contributed to the progression of liver fibrosis and cirrhosis.

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fibrrosis. In a study involving Japanese subjects with NAFLD, higher baseline hemoglobin A1c levels and a reduction in hemoglobin A1c level significantly contributed to liver fibrosis regression when treated with the sodium-glucose cotransporter 2 inhibitor, tofogliflozin. Indeed, nonalcoholic diabetic cirrhosis was identified in five postmenopausal women with diabetes before Ludwig et al introduced NASH.

These findings suggest that diabetes independently may impact NASH pathology, an effect that could be overshadowed by prevalent obesity.

We previously established two rodent models for diet-induced NASH. The first model involves the administration of methionine and choline-deficient diets to obese insulinresistant models, such as those following a high-fat diet (HFD) and obese Otsuka Long-Evans Tokushima Fatty, confirming that both inherited and acquired insulin resistance exacerbate steatohepatitis pathology. Using this model, several potential treatments were identified for steatohepatitis, including pioglitazone, tranilast, olmesartan, and metformin. This model showed NADPH oxidase-mediated reactive oxygen species generation, which may induce hepatocyte damage. The second model involves lipid-induced steatohepatitis by administering an atherogenic diet composed of cholesterol and cholic acids alongside an HFD. This model showed free cholesterol—induced oxidative stress and stellate cell activation, leading to inflammation and fibrosis. However, neither model consistently presents with diabetes.

To better understand the underlying pathology of the clinical observation that diabetes exacerbates NASH pathology, a novel mouse model of diabetic steatohepatitis was established and characterized, enabling us to explore the molecular signatures of diabetes-induced exacerbation of steatohepatitis.

Materials and Methods

Animals

All animal experiments conducted in this study were conducted in strict accordance with the guidelines and regulations set forth by the Animal Ethics Committee of Kanazawa University. The study protocol was approved by the Animal Ethics Committee (approval number: AP-132743) to ensure the ethical treatment of animals. The Animal Ethics Committee guidelines for the care and use of laboratory animals at Kanazawa University were strictly followed during the experiments. Additionally, all experiments were performed in compliance with the relevant national laws and regulations on the protection of animals. C57BL/6J male mice were obtained from Japan SLC (Shizuoka, Japan) and were housed in the Institute for Experimental Animals of Kanazawa University. The mice were maintained in a temperature-controlled environment with a 12-hour light/dark cycle. They were provided with ad libitum access to food and water under specific pathogen-free conditions. The sample sizes for each experiment are specified in the figure legends, ensuring appropriate statistical power and reproducibility.

Diets and Sample Collection

During the study, the mice were assigned to different dietary regimens. The mice in one group were fed a standard laboratory diet, MF (Oriental Yeast, Tokyo, Japan), whereas another group was fed a HFD (60% fat, D12492; Research Diets, Inc., New Brunswick, NJ). The dietary intervention lasted 8 weeks, starting from 7 weeks of age. After the 8-week experimental period, blood plasma and tissue samples were collected from the mice after a 5-hour fasting period. The samples were stored promptly at −80°C for further analysis.

Animal Experiments

The experimental design is shown in Supplemental Figure S1A. Male C57BL/6J mice, aged 7 weeks, were divided into eight groups based on the experimental diet.

Four groups received a normal diet, including the control group, carbon tetrachloride (CCL4) group, streptozotocin (STZ) group, and CCL4+STZ group. The following four groups were fed a 60% HFD, including the control group, CCL4 group, STZ group, and CCL4+STZ group.

To induce inflammation and fibrosis, the mice in the respective groups were subjected to intraperitoneal injection of CCL4 every 3 days for 8 weeks. CCL4 was mixed with olive oil at a ratio of 1:9 (v/v), and intraperitoneal injection was performed using a final concentration of 0.9 μL/g body weight.

Diabetes was induced by injecting mice intraperitoneally with STZ dissolved in sodium citrate (pH 4.5) for 7 consecutive days at a dosage of 50 mg/kg body weight. This is a well-established model of multiple low doses of STZ used to induce diabetes, and induces gradual insulinitis-mediated destruction of pancreatic β cells similarly to human type 1 diabetes, eventually leading to insulinopenic diabetes.

Animal Experiments for the Time Course Study

Male C57BL/6J mice, aged 7 weeks, were fed a 60% HFD throughout the study. Liver inflammation and fibrosis were induced by injecting CCL4 every 3 days for 4, 6, and 8 weeks, respectively, to establish mice with diabetes for 2, 4, and 6 weeks. Diabetes was induced by injecting a low dose (50 mg/kg per day) of STZ for 7 days.

Biochemical Blood Test

Blood glucose levels were measured using a GLUCOCARD G + Meter (Arkray, Kyoto, Japan). Plasma levels of aspartate aminotransferase, alanine aminotransferase,
alkaline phosphatase, lactate dehydrogenase, total cholesterol, triglycerides, and total bilirubin were analyzed by Oriental Yeast Co. (Shiga, Japan) according to their established protocols.

Hydroxyproline Content

The hydroxyproline content, which serves as an indicator of liver collagen content, was quantified using a modified version of a previously described method. 15 Liver tissue weighing 50 mg was homogenized in ice-cold phosphate-buffered saline. The homogenates then were hydrolyzed in 6 N HCl at 120°C for 16 hours. After hydrolysis, the samples were oxidized with chloramine-T at room temperature for 25 minutes. Subsequently, the reaction mixture was incubated in Ehrlich’s picrofuchsine solution at 65°C for 15 minutes. The absorbance values were measured at 540 nm after cooling the samples to room temperature.

Liver Triglyceride Content

The hepatic triglyceride content was quantified by lysing the liver tissue using 2-propanol and following the instructions provided by the manufacturer of the TG E-test kit (Wako, Osaka, Japan).

Histology

Liver tissue specimens obtained from the mice were fixed immediately in 10% neutral-buffered formalin and subsequently embedded in paraffin. Multiple 4-μm-thick sections were prepared from each paraffin-embedded block for hematoxylin and eosin staining, Masson trichrome staining, and Sirius red staining. Liver histology was scored blindly by a single pathologist for histologic evaluation. Sirius red staining was analyzed using QuPath software, and the entire positive area was analyzed across the entire slides. The degree of fatty change, hepatocellular ballooning, lobular inflammation, and fibrosis were assessed according to the criteria established by the NASH Clinical Research Network. 13

Flow Cytometry

Liver tissues from male C57BL/6 mice were mished and digested for 20 minutes at 37°C using 10 mg/mL type IV collagenase (Sigma-Aldrich) in phosphate-buffered saline containing 2% bovine serum albumin at a pH of 7.4. The resulting cell suspension was filtered and centrifuged at 2000 rpm for 3 minutes. The pellets then were resuspended in phosphate-buffered saline supplemented with 2% fetal bovine serum and incubated with Fc Block (BD Biosciences). To analyze the macrophage phenotype, fluorescence-activated cell sorting analysis was performed using the following antibodies: NK1.1-PerCP Cy5.5 (eBioscience, San Diego, CA), CD3-PerCP Cy5.5 (eBioscience), CD19-PerCP Cy5.5 (eBioscience), TER119-PerCP Cy5.5 (eBioscience), CD45-APC-Cy7 (eBioscience), Gr-1 (Ly-6G/Ly-6C)-Pacific Blue (eBioscience), F4/80-PE-Cy7 (BioLegend, San Diego, CA), CD11b-PETR (Invitrogen, Carlsbad, CA), CD11c-PE (eBioscience), and CD206-Alexa Fluor 647 (BioLegend). Dead cells were excluded by staining with propidium iodide (Sigma-Aldrich). Cell counting was performed using the BD cell viability kit (BD Biosciences). FlowJo software (Tree Star, Inc., Ashland, OR) was used for data analysis and compensation.

Quantitative PCR

Quantitative PCR was performed using the Luna Universal Quantitative PCR Master Mix (NEB, Ipswich, MA). The results of the quantitative PCR were analyzed using the Gapdh gene as an internal control and expressed as the means ± SEM in arbitrary units. The primer sequences used in this study are listed in Table 1.

Volcano Plot Visualization

Volcano plots were generated using R version 4.2.2 and the following packages: ggpubr version 0.5.0 (Kassambara A, 2023), ggplot2 version 3.4.2 (Wickham H, 2016), and ggspel version 0.9.2 (Slowikowski K, 2023). The cluster of genes in Ly6X-RM was identified by screening the genes with an adjusted P value<0.05 and a log2 fold change cutoff value>0.37 and <-0.37. The cluster of genes in liver sinusoidal endothelial cells 1 (LSEC1s) were identified by screening genes with an adjusted P value<0.05 and a log2 fold change cutoff value>0.37 and <-0.37.

Single-Cell RNA Sequencing

Cell Preparation and Flow Cytometry

Liver cells from adult male C57BL/6J mice were isolated based on previously described. 14 In brief, mice were euthanized, and their livers were excised, minced, and subsequently subjected to enzymatic digestion at 37°C with continuous shaking. The digestion process involved sequential treatment with the following enzymes: dispase II (cat. 17105-041; Gibco) supplemented with DNase I (cat. 0293; Invitrogen, Carlsbad, CA), collagenase (cat. 034-22363; Wako), and collagenase type 2 (cat. LS004176; Worthington Biochemical Corp., Lakewood, NJ). After enzymatic and mechanical dissociation, the sample was filtered through a 40-μm cell strainer (cat. 352340; Falcon) to obtain a single-cell suspension. Red blood cells were lysed with ammonium-chloride-potassium buffer (0.15 mol/L NH4Cl, 10 mmol/L KHCO3, and 0.1 mmol/L Na2-EDTA). For the staining process, 0.5 μg Fc blocking antibody (purified rat anti-mouse CD16/CD32 antibody), CD31-BV 421
Table 1 Primers for Quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Gapdh</td>
<td>5'-TGACACCACTGCCATTTAACG-3'</td>
<td>5'-GGCATGGACTGTGGTCATAG-3'</td>
</tr>
<tr>
<td>Hgf</td>
<td>5'-TCTCCCTTTGTTTTGGG-3'</td>
<td>5'-CTTCTTTTGTGGG-3'</td>
</tr>
<tr>
<td>I6</td>
<td>5'-CCTACTCAAGCAGGAGGCTAA-3'</td>
<td>5'-GCAAGTCACTATCTTCTATAC-3'</td>
</tr>
<tr>
<td>Tnf</td>
<td>5'-AACGCCTGAGCCCCACGTGTA-3'</td>
<td>5'-GGCACACTAGTGGTTCTTCTTG-3'</td>
</tr>
</tbody>
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These data from the two groups then were integrated using the IntegrateData function.

After integration, the ScaleData function was used to calculate Z-scores and regressed out unwanted variation arising from cell-cycle differences. To reduce the data dimensionality, principal component analysis was executed on the identified variable features. Nearest neighbors were located, and clustering was performed at a specified resolution (resolution = 0.5).

The integrated data set was visualized via dimensionality reduction techniques, including principal component analysis and uniform manifold approximation and projection. The resulting integrated clusters were correlated with the original data sets. Finally, differential gene expression was assessed using the FindMarker function within the Seurat package.

Statistical Analysis

Two-way analysis of variance followed by the Tukey multiple comparisons test was performed as the post hoc test to determine significant differences. Statistical significance was defined as P < 0.05. GraphPad Prism version 9.3.0 (GraphPad Software, La Jolla, CA) was used for all data analysis.

Results

STZ-Diabetes Induces Nodular Liver without Enhancing Liver Fibrosis

The 7-week-old C57BL/6J male mice were fed an HFD and subjected to CCl₄ injection every 3 days for 8 weeks to induce liver inflammation and fibrosis. Diabetes was induced by injecting a low dose (50 mg/kg per day) of STZ for 7 consecutive days (Supplemental Figure S1A). STZ-induced diabetes for 6 weeks significantly reduced body weight and increased blood glucose levels in the mice (Figure 1, A and B; Supplemental Figure S1, B and C).

In normal diet-- and HFD-fed mice treated with and without CCl₄, STZ-induced diabetes resulted in significant liver enlargement (Figures 1C and 2A), increased liver weight--to--body weight ratios, increased liver triglyceride contents (Figure 1, C and D), higher steatosis scores (Figure 3, A–E), and the formation of liver nodules (Figure 2, A–C). CCl₄ treatment significantly increased scores for liver inflammation, hepatocyte ballooning,
Figure 1  Anthropometry and blood biochemistry in high-fat diet (HFD), carbon tetrachloride (CCL₄), and streptozotocin (STZ)-treated mice. A: Body weight of mice after 5 hours of fasting. B: Blood glucose levels of mice after 5 hours of fasting. C: Liver weight-to-body weight ratio of the mice. D: Liver triglyceride content. E: Plasma levels of aspartate aminotransferase (AST). F: Liver hydroxyproline content. All mice were from the 6WD model. ND group: control, n = 7; CCL₄, n = 8; STZ, n = 16; and CCL₄+STZ, n = 11; HFD group: control, n = 7; CCL₄, n = 6; STZ, n = 15; and CCL₄+STZ, n = 8. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. 6WD, 6-week diabetic mice; ND, normal diet.
Figure 2  Representative morphologic and histologic features of the liver. **A:** Liver appearance. **B:** Hematoxylin and eosin staining. **C:** Masson trichrome staining. **D:** Representative images of Sirius red staining for quantitative analysis. The pixel size used in positive-area analysis was 0.88 μm/pixel, and the pixel size used in the whole slide image analysis was 0.44 μm/pixel. Scale bars: 1 cm (**A** and **C**); 100 μm (**B**). CCl₄, carbon tetrachloride; HFD, high-fat diet; ND, normal diet; STZ, streptozotocin.
hepatocellular damage, fibrosis (Figure 3, A–E), and the positive rates of Sirius red staining (Figures 2D and 3F). STZ-induced diabetes significantly enhanced CCl₄-mediated hepatocyte ballooning and fibrosis (Figure 3, A–E), without enhancing the CCl₄-mediated inflammation (Figure 3, F).

To evaluate the histologic time course of multinodular formation induced by STZ-induced diabetes on HFD+CCl₄, the liver was examined at different time points (Supplemental Figure S2). STZ-induced diabetes for 2 or 4 weeks significantly increased liver weight (Supplemental Figure S2, C and D) and enhanced steatosis (Supplemental Figure S2, E and F), which became less evident at 6 weeks of diabetes, indicating burn-out steatohepatitis. STZ-induced diabetes did not affect CCl₄-mediated inflammation but significantly enhanced CCl₄-mediated...
hepatocyte ballooning (Supplemental Figure S2, E and F). CCl₄ treatment increased the positive rates of Sirius red staining, which were reduced by STZ-induced diabetes over the course (Supplemental Figure S2, G and H).

These time course findings suggest that STZ-induced diabetes enhances hepatocyte ballooning from early stages and finally enhances liver fibrosis and nodular formation.

Effects of CCl₄ and STZ-Induced Diabetes on Blood and Liver Biochemistry

CCl₄ treatment increased plasma levels of liver enzymes, such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase, in the mice fed the HFD, but not the normal diet (Figure 1E; Supplemental Figure S1, D–F). CCl₄ reduced plasma levels of total cholesterol, which were restored by STZ-induced diabetes in HFD-fed mice (Supplemental Figure S1G). Combined CCl₄+STZ-induced diabetes significantly increased plasma levels of triglycerides and total bilirubin in HFD-fed mice (Supplemental Figure S1, H and I). CCl₄ treatment significantly increased liver hydroxyproline contents, which were unaffected by STZ-induced diabetes (Figure 1F).

STZ-Induced Diabetes Regulates M1/M2 Macrophage Polarization in the Liver

STZ-induced diabetes significantly up-regulated the representative genes involved in liver regeneration (Hgf) and inflammation (Il6 and Tnf) in the livers of HFD-fed mice (Figure 4, A–C).

Because Il6 and Tnf are expressed in macrophages, macrophage polarization in the liver was examined using flow cytometry analyses (Figure 5). CD11c+M1-like macrophages and CD206+M2-like macrophages were gated and counted separately (Supplemental Figure S3A). In HFD-fed mice treated with CCl₄, STZ-induced diabetes increased the percentage of M1 macrophages (Figure 5B), reduced the percentage of M2 macrophages (Figure 5C), and, consequently, increased the M1/M2 ratios in the liver (Figure 5D). Total macrophage numbers in the liver also were analyzed, considering variations in liver weight and cell damage among the treatment groups (Supplemental Figure S3, B–E). In HFD-fed mice treated with CCl₄, the STZ-induced diabetes decreased total M2 macrophage numbers in the whole liver and M2 macrophage numbers per gram of liver, but did not affect M1 macrophage numbers (Supplemental Figure S3, B–E). These findings suggest that STZ-induced diabetes reduces M2 macrophages in the liver.

scRNAseq Signatures in Diabetic Steatohepatitis

To investigate the involvement of resident cells in alleviating steatohepatitis, scRNAseq analyses were conducted, focusing primarily on nonparenchymal cells, in the HFD+CCl₄ (nondiabetic) and HFD+CCl₄+STZ (diabetic) groups. scRNAseq analysis revealed unique cell clusters altered between the steatohepatitis models with and without diabetes (Figure 6A). Macrophages were classified into four clusters: recruited macrophages occupying the NASH Kupffer cell niche, Ly6C-high recruited macrophages (Ly6X-RM), Kupffer cells, and Ly6C-low recruited macrophages. STZ-induced diabetes reduced the residential macrophage Kupffer cells while increasing neutrophils and bone marrow–derived recruited inflammatory macrophages, such as recruited macrophages occupying the NASH Kupffer cell niche and Ly6X-RM (Figure 6B). On the contrary, STZ-induced diabetes globally reduced LSECs, natural killer cells, and B cells (Figure 6B).

Given the significant alterations in LSEC1 and Ly6X-RM as a result of diabetes, coordinated up-regulated pathways and genes in Ly6X-RM (Figure 7, A and C) and LSEC1 (Figure 7, B and D) of the STZ diabetic group were extracted. In Ly6X-RM, genes involved in the inflammatory response (Sl100a8, S100a9, Ccl2, Igb3, Icam1, Lyve1, Retnlg, and S1pr1) and the apoptotic signaling pathway (Bax, Cebpb, Ddit4, Cdkn1a, Eda2r, Ihhbb, Phlda3, and Tnfaip3) were coordinately up-regulated (Figure 7, A and C; Supplemental Figure S4A). In LSEC1, genes involved in leukocyte migration (Sl100a8, S100a9, Ccl2, Igb3, Icam1, Lyve1, Retnlg, and S1pr1) and the apoptotic signaling pathway (Bax, Cebpb, Ddit4, Cdkn1a, Eda2r, Ihhbb, Phlda3, and Tnfaip3) were coordinately up-regulated (Figure 7, B and D; Supplemental Figure S4, B and C).

In the stellate cell cluster, genes involved in myofibroblast formation/fibrosis (Col1a1, Col4a1, Col4a2, Acta2, Tgb1, Tgb2, Pdgfa, Pdgfrb, Mmp2, and Timp1) were down-regulated in the HFD+CCl₄+STZ (diabetic) group compared with the HFD+CCl₄ (nondiabetic) group (Figure 4D) in concert with the hepatic hydroxyproline contents (Figure 1F), Sirius red staining (Figures 2D)...

Discussion

Our study discovered that diabetes exacerbates the pathology of HFD and CCl₄-mediated steatohepatitis in mice. Specifically, diabetes enhanced hepatocyte degeneration, followed by regenerative nodule formation. This model provides the first experimental evidence of diabetes aggravating the progression of steatohepatitis independently of obesity.

Our present diabetic steatohepatitis model features extreme insulin deficiency to discriminate hyperglycemia and insulin secretory failure from obesity in the pathophysiology of type 2 diabetes. Type 2 diabetes is caused by impaired insulin action resulting from insulin resistance and impaired compensatory insulin secretion. The insulin-secretory capacity against insulin resistance varies among ethnic groups. Asian people have an impaired insulin-secretory capacity and develop diabetes at a lower body...
Figure 4  Hepatic expression of the genes involved in liver regeneration, inflammation, and fibrosis. A–C: Fold change of mRNA levels of Hgf, Il6, and Tnf in control, carbon tetrachloride (CCL4) streptozotocin (STZ), and CCL4+STZ mice in both the normal diet (ND) and high-fat diet (HFD) groups. D: Violin plot of genes involved in myofibroblast formation/fibrosis in the stellate cell cluster were as follows: Col1a1, Col4a1, Col4a2, Acta2, Tgfb1, Tgfb2, Pdgfa, Pdgfrb, Mmp2, and Timp1. All mice were from the 6WD model. ND group: control, n = 7; CCL4, n = 8; STZ, n = 16; and CCL4+STZ, n = 11; HFD group: control, n = 7; CCL4, n = 6; STZ, n = 15; and CCL4+STZ, n = 8. *P < 0.05, **P < 0.01, and ****P < 0.0001.
Figure 5  Diabetes shifts M1/M2 macrophage polarization in the liver. A: Representative flow cytometry plots. B: Percentage of M1 macrophages. C: Percentage of M2 macrophages. D: M1 macrophage to M2 macrophage ratios. All mice were from the 6WD model. Normal diet (ND) group: control, n = 7; carbon tetrachloride (CCl₄), n = 8; streptozotocin (STZ), n = 16; and CCl₄ + STZ, n = 11; high-fat diet (HFD) group: control, n = 7; CCl₄, n = 6; STZ, n = 15; and CCl₄ + STZ, n = 8. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
mass index and insulin resistance than Caucasian people. Thus, insulin treatment often is required for ameliorating hyperglycemia and its associated liver damage in Asian patients with diabetes. Based on these backgrounds, lean NAFLD is relatively common in Japan. Previous clinical observations in Japanese populations have indicated that diabetes independently exacerbates the pathology of chronic hepatitis C and NAFLD. In the natural course chronic hepatitis C after transfusion, type 2 diabetes independently accelerates liver fibrosis and the subsequent development of hepatocellular carcinoma and mortality. Obesity alone does not accelerate the histologic course of the liver, but accelerates it as an added factor in the presence of diabetes. In serial liver biopsy studies in Japanese

Figure 6  Single-cell RNA sequencing signatures in diabetic steatohepatitis. A: Uniform manifold approximation and projection (UMAP) plot of a total of 9793 single cells from mouse livers. Colors represent T cells, B cells, natural killer (NK) cells, Kupffer cells, recruited macrophages occupying the NASH Kupffer cell niche (KN-RM-NASH), Ly6C-high recruited macrophages (Ly6C<sup>Hi</sup>-RM), Ly6C-low recruited macrophages (Ly6C<sup>Lo</sup>-RM), liver sinusoidal endothelial cells 1 (LSEC1), liver sinusoidal endothelial cells 2 (LSEC2), portal endothelial cells (Portal EC), erythroid cells, plasma cells, stellate cells, neutrophils, myofibroblasts, and cholangiocytes. B: Fraction of cells in each cluster. CCl<sub>4</sub>, carbon tetrachloride; HFD, high-fat diet; KC-N, ____; STZ, streptozotocin.
Figure 7  Genes and pathways altered significantly in Ly6C-high recruited macrophages (Ly6Ch-RM) and liver sinusoidal endothelial cells 1 (LSEC1) clusters of the liver. A and B: Volcano plot of gene expression changes in Ly6C-RM (A) and in LSEC1 (B) between high-fat diet (HFD)+carbon tetrachloride (CCl₄) and HFD+CCl₄+streptozotocin (STZ) groups [with log₂ fold change (FC) over ±0.37]. Blue indicates down-regulated genes, and red indicates up-regulated genes. The two vertical black dashed lines are the log₂ fold change cut-off values (>0.37 and <-0.37) and the horizontal black dashed line is the adjusted P value <0.05. The left upper part of the volcano plot with the genes in blue indicates those genes are down-regulated under the criteria log₂FC < -0.37 and adjusted P value <0.05. The central upper part of the volcano plot includes genes in gray that do not present an alteration in their expression (log₂FC between -0.37 and 0.37), but have an adjusted P value <0.05. The right upper part of the volcano plot shows genes in red that are considered up-regulated by the following conditions, log₂FC > 0.37 and adjusted P value <0.05. The genes under the horizontal black dashed lines represent the genes that are considered to have an unchanged expression and are not statistically significant (adjusted P value >0.05). C: Gene ontology (GO) analysis of differentially expressed genes (DEGs) in Ly6C-RM of the HFD+CCl₄+STZ group (up-regulated genes with log₂ fold change >0.37). The top 15 GO terms. D: GO analysis of DEGs in LSEC1 of the HFD+CCl₄+STZ group (up-regulated genes with log₂ fold change >0.37). The top 15 GO terms. The 2-week diabetic mouse model was used for the single-cell RNA sequencing analyses.
subjects with NAFLD and type 2 diabetes, a reduction in hemoglobin A1c level, rather than weight, alleviates liver fibrosis.\textsuperscript{5,16} In addition, serum insulin-like growth factor-1 (IGF-1) levels are lower in patients with uncontrolled type 2 diabetes and impaired insulin secretion,\textsuperscript{27} suggesting that diabetes impairs growth hormone (GH)–IGF-1 signaling. Fatty liver and steatohepatitis are more frequent in patients with adult GH deficiency than in those without GH deficiency.\textsuperscript{21,22} Indeed, GH replacement therapy reverses NASH in patients with adult GH deficiency.\textsuperscript{21,23} In addition, a GH-deficient rat model exhibited steatosis, hepatocyte injury, and fibrosis in the liver, which were restored by GH and IGF-I administration.\textsuperscript{24} Reduced IGF-1 production in the liver with advanced NASH could exacerbate liver fibrosis further. These findings suggest possible hepatoprotective effects of insulin–IGF-1 signaling in the pathology of NASH, which are impaired in insulinopenic diabetes. Based on these clinical observations, the diabetic steatohepatitis model in the present study was developed to address the impacts of hyperglycemia and insulin secretory failure on liver pathology.

It is important to note that a similar NASH model incorporating diabetes, known as the STAM model, has been reported and is commercially available\textsuperscript{25} (SMC Laboratories, Inc., Tokyo, Japan). The STAM model induces diabetes in male mice through a single subcutaneous injection of 200 μg STZ 2 days after birth, followed by HFD feeding from 4 weeks of age. These mice exhibit hyperglycemia, hepatomegaly, and the development of characteristic histologic features associated with NASH over time.\textsuperscript{25} The current model combines HFD, CCl4, and STZ to induce steatohepatitis and regenerative nodule formation at specific time points. Although both models feature nodular liver development without cirrhosis, this study is the first to directly compare the impact of diabetes itself on liver pathology with nondiabetic models. This study showed that diabetes exacerbates liver degeneration, regenerative nodular formation induced by HFD and CCl4, and the up-regulation of genes involved in liver regeneration.

Interestingly, this study observed that diabetes did not enhance liver fibrosis during the course. Indeed, genes involved in myofibroblast formation and fibrosis were down-regulated in the diabetic groups in the stellate cell cluster. Masson trichrome staining may overestimate liver fibrosis because it is positive not only for collagen fiber but also for preexisting reticulin fiber aggregated by hepatocellular necrosis. In addition, fibrosis scoring based on Masson staining tends to overestimate fibrosis status compared with quantitative evaluation because it is based on the presence or absence of a single finding, such as fibrosis, fibrotic septa, or regenerative nodules. In contrast, Sirius red staining detects collagen fiber specifically, extracts fiber regions with color information, and, therefore, is used widely as an excellent staining method for quantification analyses. This study shows that diabetes does not enhance liver fibrosis during the course despite the severe regenerative nodule formation in this model. Instead, the primary pathology in the diabetic liver is extensive cellular damage reflected by hepatocyte ballooning. The formation of regenerative nodules likely is attributed to pervasive cell death in diabetes. It is speculated that the liver damage induced by diabetes in this model may have been too severe to be compensated by liver fibrosis. This hypothesis warrants further investigation in future studies.

Because STZ-induced diabetes up-regulated genes involved in inflammation, such as Il6 and Tnf, in the liver, the macrophage polarity\textsuperscript{26} affecting inflammatory response in the liver was examined. This study found that diabetes influenced the polarization of macrophages in the liver. Specifically, diabetes increases M1 macrophages and reduced M2 macrophages involved in anti-inflammatory and tissue repair processes. These alterations in macrophage polarity may contribute to the inflammation observed in steatohepatitis. Although the role of hyperglycemia on macrophage polarity, particularly in the liver, has been less explored, studies have shown that hyperglycemia can promote M1 polarization and inhibit M2 polarization of liver resident Kupffer cells, exacerbating liver injury.\textsuperscript{27,28} This study provides further evidence that diabetes polarizes macrophages toward an inflammatory phenotype in the liver of mice fed an HFD and treated with CCl4.

To investigate the cell–cell interactions responsible for diabetes-associated inflammation, scRNAseq analyses focusing on nonparenchymal cells were conducted. The findings showed that diabetes reduced the number of residual macrophages, specifically Kupffer cells, while increasing the presence of bone marrow–derived recruited macrophages, such as Ly6X-RM. The up-regulation of genes involved in the inflammatory response, including S100a8 and S100a9, was observed in Ly6X-RM. Notably, these genes encode ligands for receptors for advanced glycation endproducts (RAGE) \textsuperscript{29}Toll-like receptor 4 (TLR4) and RAGE-target genes, such as Ccl2, Icam1, and Bax,\textsuperscript{31,32} were among the up-regulated genes in LSECs. These findings suggest that diabetes may up-regulate RAGE/Toll-like receptor 4 ligands in activated recruited inflammatory macrophages and impair LSECs, potentially promoting leukocyte recruitment/adhesion and inducing apoptosis. Because LSECs act as a platform for the adhesion of Kupffer cells and stellate cells,\textsuperscript{33} LSEC injury may trigger the pathology of steatohepatitis. Indeed, a recent serial liver biopsy study showed that genes involved in LSECs in zone 3 were coordinately down-regulated with the progression of liver fibrosis.\textsuperscript{19} In addition, in an intervention study in subjects with type 2 diabetes and NAFLD, genes involved in LSECs were coordinately down-regulated in the liver with severe steatosis, which was recovered with treatment with sodium-glucose cotransporter 2 inhibitor tofosfalozin.\textsuperscript{3}
In conclusion, this study established a novel mouse model of diabetic steatohepatitis by combining HFD, CCl₄, and STZ. Diabetes exacerbated the development of steatosis, hepatocyte ballooning, regenerative nodular formation, and macrophage M1/M2 ratios in this model. These scRNAseq analyses suggest that diabetes activates inflammatory recruited macrophages and impairs LSECs through the RAGE/Toll-like receptor 4 signaling pathway, providing potential therapeutic targets for treating hepatocyte degeneration in diabetic steatohepatitis. Based on these findings, it is proposed that diabetic steatohepatitis may be recognized as a diabetic vascular complication, and further investigations are warranted to explore the underlying molecular links between diabetic vascular damage and steatohepatitis.

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Author Contributions

T.A., T.T., Y.Y., K.K., and Y.O. conceived the study and designed the experiments; T.A., T.I., K.K., Y.O., H.G., K.K., N.L., H.A., and Q.F.L. conducted the experiments; T.A., T.T., Y.Y., K.K., and Y.O. performed the analyses; and T.A. and T.T. wrote and edited the manuscript with input from the other authors.

Disclosure Statement

None declared.

Supplemental Data

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References
