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Strain-Dependent Effects of Dietary Cholic Acid on Liver Fibrogenesis and Gut Microbiota in TSNO and TSOD Mice

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Abstract

Background: Metabolic dysfunction-associated steatohepatitis (MASH) is defined by hepatocellular damage accompanied by inflammation and fibrotic changes. Bile acids (BAs) and gut microbiota play pivotal roles in disease progression. However, the contribution of dietary cholic acid (CA), a primary BA, remains unclear. **Methods:** We investigated the effect of dietary CA supplementation in Tsumura–Suzuki obese diabetic (TSOD) and Tsumura–Suzuki non-obese (TSNO) mouse strains with distinct metabolic phenotypes. The mice were fed normal diet (ND) or 0.5% CA-supplemented ND. Liver injury, fibrosis, and macrophage dynamics were assessed by biochemical assays, histology, flow cytometry, and RT-qPCR. Gut microbiota composition and fecal BA profiles were analyzed using 16S rRNA sequencing and mass spectrometry. **Results:** CA supplementation induced hepatomegaly, liver injury, and lipid metabolism abnormalities in both strains. In TSNO mice, CA markedly enhanced hepatic fibrosis, increased *Col1a1* and *Timp1* expressions, and promoted CD11c⁺ monocyte-derived macrophage infiltration. In contrast, TSOD mice showed minimal fibrotic responses to CA but exhibited pronounced alterations in gut microbiota composition, including enrichment of *Akkermansia muciniphila*, along with changes in fecal BA profiles. Flow cytometry further revealed Kupffer cell numbers and increased macrophage recruitment in both strains after CA supplementation. **Conclusions:** Dietary CA exerts strain-dependent effects on MASH pathogenesis. CA promoted macrophage-driven hepatic fibrosis in TSNO mice, whereas it primarily modulated gut microbiota and BA metabolism in TSOD mice. These findings highlight the dual roles of CA in linking hepatic immune responses with intestinal homeostasis and suggest a context-dependent contribution to MASH progression.

Keywords: bile acid; cholic acid; gut microbiota; macrophage; metabolic dysfunction-associated steatohepatitis



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1. Introduction

Metabolic dysfunction-associated steatohepatitis (MASH) has increasingly been regarded as a major issue for global health, reflecting the rapid increase in type 2 diabetes mellitus (T2DM) worldwide [1,2]. It is characterized by inflammation, hepatocellular injury, and fibrosis, which represent major factors associated with an increased risk of cirrhosis and hepatocellular carcinoma [3,4].

Among the immune cell populations involved, hepatic macrophages are increasingly recognized as central mediators of disease progression. Resident Kupffer cells (KCs) maintain homeostasis under physiological conditions, whereas bone marrow-derived monocyte-derived macrophages (MdMs) infiltrate the liver during injury and orchestrate pro-inflammatory and fibrogenic responses [5,6]. A distinct CD11c⁺ macrophage subset forms hepatic crown-like structures (hCLS), in which immune cells surround dead hepatocytes, a feature closely associated with fibrogenesis in both experimental models and human MASH [7,8]. Conversely, Ly6C⁺ macrophages are involved in tissue repair and inflammation resolution [9], highlighting the functional heterogeneity of hepatic macrophages.

In addition to cellular mediators, bile acids (BAs) are signaling molecules that connect metabolic regulation and inflammation. Acting through the farnesoid X receptor (FXR) and TGR5, BAs regulate lipid and glucose metabolism, BA synthesis, and immune cell functions [10,11]. Among primary bile acids, cholic acid (CA) shows particularly paradoxical effects, improving glucose metabolism while, in certain contexts, promoting hepatic lipid accumulation and hepatotoxicity [12]. Moreover, BAs and the gut microbiota form a dynamic interplay in which the BA composition shapes microbial communities, and the microbial metabolism of BAs feeds back to regulate host physiology [13,14]. *Akkermansia muciniphila*, for example, protects against obesity and fatty liver disease by modifying the gut barrier integrity, BA metabolism, and host immune response [15,16]. These findings emphasize the impact of gut–liver axis in MASH pathogenesis.

Rodent models are indispensable for elucidating the cellular and molecular mechanisms underlying MASH [17]. In this context, Tsumura–Suzuki obese diabetes (TSOD) and Tsumura–Suzuki non-obese (TSNO) mouse strains provide unique opportunities to investigate the contribution of host metabolic background. TSNO mice develop advanced bridging fibrosis with characteristic macrophage infiltration when fed a high-fat, cholesterol, and cholate (iHFC) diet [18]. Using this model, we have previously demonstrated that CD11c⁺/Ly6C[−] macrophages promote fibrogenesis, whereas CD11c[−]/Ly6C⁺ macrophages contribute to anti-inflammatory responses [19]. In contrast, TSOD mice spontaneously develop obesity, insulin resistance, and T2DM, accompanied by distinct alterations in gut microbiota and BA metabolism [20]. Collectively, these findings suggest that the TSNO and TSOD mice provide complementary insights into the immunometabolic mechanisms that drive MASH.

We have previously demonstrated that feeding TSNO mice an iHFC diet lacking CA revealed the pivotal role of CA in MASH progression [21]. Specifically, CA promotes hepatic MdM accumulation and modulates gut microbiota composition and BA profiles, thereby contributing to disease progression [21]. Furthermore, in TSOD mice, the iHFC diet improved T2DM-related metabolic abnormalities, an effect associated with CA-mediated gut microbiota modulation, particularly *A. muciniphila* expansion [22]. However, the direct roles of dietary CA in regulating macrophage dynamics, hepatic fibrogenesis, and gut–liver interactions in these models remain to be fully elucidated. This study explored the effects of CA supplementation in TSOD and TSNO mice fed with a normal diet (ND) by integrating histological, immunological, and microbiome analyses to elucidate the strain-dependent mechanisms by which CA contributes to MASH progression.

2. Materials and Methods

2.1. Animal Studies

All experimental procedures involving animals, including the study design, statistical analysis, and animal care, were carried out in compliance with the ARRIVE guidelines and the guidelines for the Proper Conduct of Animal Experiments issued by the Science Council of Japan. The study protocols received approval from the Ethics Committee for Animal Experiments at Toyama Prefectural University (Approval no. R1-3, R4-1, and R5-21).

Male TSOD and TSNO mice at six weeks of age were purchased from the Institute of Animal Reproduction (Ibaraki, Japan) and maintained under specific pathogen-free conditions with free access to water and food and a 12 h light/dark cycle. Mice were randomly allocated to each experimental group. Histological evaluation and scoring were conducted by K.T. and M.I.-S. in a blinded fashion, with the investigators unaware of the group assignments during outcome assessment. From seven weeks of age, the mice were fed ND from Oriental-Yeast (Tokyo, Japan) or ND supplemented with 0.5% (*w/v*) CA (FUJIFILM Wako Pure Chemical, Osaka, Japan). The concentration of cholic acid (0.5%) was selected to match the cholic acid content of the iHFC diet that we have previously used to induce MASH in TSNO and TSOD mice, allowing direct comparison with our earlier studies [19–22]. At the end of the experimental period, the animals were placed under isoflurane anesthesia (FUJIFILM Wako Pure Chemical), and blood and liver samples were collected for subsequent analysis.

2.2. Plasma Chemistry

Blood collection was performed via the inferior vena cava, after which plasma was isolated by centrifugation. Levels of alanine aminotransferase (ALT), total cholesterol (T-CHO), triglycerides (TG), total bilirubin (T-Bil), γ -glutamyltransferase (GGT), and alkaline phosphatase (ALP) in plasma were measured using a DRI-CHEM NX700 analyzer (FUJIFILM, Tokyo, Japan).

2.3. Non-Parenchymal Cell Isolation

For isolation of hepatic non-parenchymal cells, the mice were anesthetized with isoflurane and perfused with PBS (Nacalai Tesque, Kyoto, Japan). A liver Dissociation Kit was used for non-parenchymal cells isolation (Miltenyi Biotec, Bergisch Gladbach, Germany). After filtration through a 100 μ m cell strainer, the cell suspension was used for flow cytometric analysis.

2.4. Flow Cytometry

To prevent nonspecific Fc γ R-mediated binding, non-parenchymal cells (1.25×10^5) were preincubated with an anti-Fc γ R antibody (clone 2.4G2; BD Biosciences, CA, USA). After 20 min, the cells were stained with optimal concentrations of each antibody. Non-viable cells were excluded using 7-amino-actinomycin D (7-AAD). Flow cytometric was performed using a FACSCanto II instrument (Becton Dickinson, Franklin Lakes, NJ, USA), and data were processed with FlowJo software Version 10.8 (BD Biosciences). A list of antibodies is provided in Supplementary Table S1.

2.5. Quantitative Real-Time PCR

A NucleoSpin RNA Mini Kit was used to isolate total RNA (Macherey-Nagel, Düren, Germany). The extracted RNA was reverse-transcribed using the PrimeScript[®] RT reagent kit (Takara Bio Inc., Shiga, Japan). Quantitative RT-PCR was performed with FastStart Universal Probe Master (Roche Applied Science, Mannheim, Germany). Amplification was monitored using the CFX96 Touch[™] Real-Time PCR Detection System (Bio-Rad, Hercules,

CA, USA). Gene expression levels were normalized to those of Hprt mRNA, which was used as an internal reference. Details of the TaqMan probes (Applied Biosystems, Waltham, MA, USA) are shown in Supplementary Table S2.

2.6. Liver Histology and Immunohistochemistry

Liver samples were fixed in 4% formaldehyde. After fixation, tissues were embedded in paraffin, cut into 6 μ m sections, and placed on glass slides. Sections were stained with hematoxylin and eosin (H&E) or Sirius Red using conventional procedures. Histological scoring was carried out using the NASH Clinical Research Network Scoring System [23]. The NAFLD activity score (NAS) was defined as the unweighted sum of the scores for steatosis, lobular inflammation, and hepatocyte ballooning and ranged from 0 to 8. An NAS of 0 to 2 was regarded as non-diagnostic of steatohepatitis, whereas scores of 5 or higher were considered diagnostic of steatohepatitis [23]. The antibody against F4/80 was acquired from Cedarlane Laboratories (Burlington, ON, Canada). Positive areas for Sirius Red and F4/80 were quantified using ImageJ software (version 1.53t) [24].

2.7. Metagenomic 16S rRNA Sequencing

Fecal DNA was isolated as reported previously [20]. Libraries for 16S rRNA gene sequencing were constructed according to the Illumina protocol (San Diego, CA, USA), as reported in an earlier study. The libraries were pooled and sequenced using a MiSeq System with a 500-cycle kit (Illumina).

2.8. Bacterial Community Analysis

Microbials composition was examined using the Quantitative Insight Into Microbial Ecology 2 (QIIME2) Ver.2021.2 pipeline [20]. Sequence reads were transformed into amplicon sequence variants (ASVs) using the DADA2 algorithm. To evaluate α - and β -diversity, a core-metrics-phylogenetic diversity analysis was carried out at a sampling depth of 10,000 reads. Taxonomic classification was carried out using the SILVA138 reference database.

2.9. qPCR Analysis for Relative Abundance of *A. muciniphila*

RT-qPCR was carried out using *A. muciniphila*-specific primers (Forward: CAG CAC GTG AAG GTG GGG AC, Reverse: CCT TGC GGT TGG CTT CAG AT), THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan), and a CFX Connect Real-Time System (Bio-Rad).

2.10. Bile Acid Analysis

Fecal BAs were extracted by homogenizing dried fecal samples (30–60 mg) in a solution containing 0.5 mL methanol, 0.8 mL acetonitrile, and 0.2 mL 28% (*w/v*) ammonium hydroxide, with 100 nmol 23-dinor-deoxycholic acid added as an internal standard (Steraloids, Newport, RI, USA). The homogenates were centrifuged, and the resulting supernatants were subjected to solid-phase extraction using appropriate columns to isolate BA-containing fractions. Fecal BAs were quantified by liquid chromatography–electrospray ionization–mass spectrometry.

2.11. Statistical Analysis

Statistical significance was assessed using two-way ANOVA, followed by Tukey's post hoc test for multiple comparisons, or an unpaired Student's *t*-test for comparisons between groups. Analyses were carried out using GraphPad Prism 9 (GraphPad, Boston, MA, USA). An F-test was carried out in Prism 9 to confirm that the *p*-value from the F-test was greater than 0.05, indicating homogeneity of variance. *p* < 0.05 was considered statistically significant. Data are presented as the mean $\pm$ standard deviation.

3. Results

3.1. Dietary CA Induces Hepatomegaly, Liver Injury, and Lipid Metabolism Abnormalities in TSOD and TSNO Mice

We first examined the role of CA-induced changes in body weight, liver size, hepatic injury, and lipid-related parameters. In the TSNO mice, no significant differences in body weight were observed between the groups (Figure 1A, left). However, the ND-fed TSOD mice exhibited significantly higher body weights than the ND-fed TSNO mice (Figure 1A, left). Furthermore, the body weight of the ND-fed TSOD mice was significantly higher than that of the ND+CA-fed TSOD mice (Figure 1A, left). Food intake tended to be lower in TSOD mice than in TSNO mice; however, no significant differences were observed between the groups (Figure 1A, right). Liver weight was significantly higher in ND+CA-fed mice than in ND-fed mice, although no significant inter-strain differences were observed under the same diet (Figure 1B). Macroscopically, ND+CA-fed TSNO mice exhibited more hepatomegaly than ND-fed TSNO mice, and the livers of ND+CA-fed mice appeared paler than those of ND-fed mice after 8 weeks (Figure 1C). Plasma ALT levels were higher in ND+CA-fed TSNO mice than in ND-fed mice after 4 weeks; however, prolonged feeding did not further increase these levels (Figure 1D). Interestingly, plasma ALT levels differed significantly between ND- and ND+CA-fed TSOD mice after 8 weeks (Figure 1D). No significant differences were found in the plasma T-CHO levels among the groups (Figure 1E, left). Plasma TG levels were significantly lower in the ND+CA-fed TSNO mice than in the ND-fed mice (Figure 1E, middle panel). However, ND-fed TSOD mice exhibited lower TG levels than ND-fed TSNO mice at 8 weeks (Figure 1E, middle panel). Plasma T-Bil levels were significantly higher in ND-fed TSOD mice than in ND-fed TSNO mice (Figure 1E, right). These findings indicated that the addition of CA to ND induced hepatomegaly, liver injury, and lipid metabolism abnormalities in both strains, with a more pronounced effect observed in TSNO mice.

We also analyzed plasma GGT and ALP levels in ND-fed and ND+CA-fed TSNO and TSOD mice. In TSNO mice, plasma GGT levels were significantly higher in the ND+CA-fed group than in the ND-fed group at 4 weeks (Figure 1F, left). In contrast, plasma GGT levels in TSOD mice were not significantly different between the ND-fed and ND+CA-fed groups at either time point (Figure 1F, left). Plasma ALP levels were also significantly increased in ND+CA-fed TSNO mice compared with ND-fed TSNO mice at both 4 and 8 weeks (Figure 1F, right). Under ND-fed conditions, TSOD mice exhibited significantly higher plasma ALP levels than TSNO mice at both time points (Figure 1F, right). However, plasma ALP levels in TSOD mice were not significantly different between the ND-fed and ND+CA-fed groups at either 4 or 8 weeks (Figure 1F, right). These results indicate a strain-dependent difference in plasma GGT and ALP responses to dietary CA in TSNO and TSOD mice.

3.2. Dietary CA Promotes Fibrotic Change in the Liver of TSNO Mice

Histological analysis of liver sections stained with H&E after 8 weeks of feeding revealed that CA supplementation significantly induced fibrosis in TSNO mice (Figure 2A,B). Fibrosis was slightly more pronounced in ND-fed TSOD mice than in ND-fed TSNO mice; however, no apparent changes were observed upon CA supplementation (Figure 2B). In contrast, none of the ND+CA-fed mouse strains showed greater steatosis, lobular inflammation, or hepatocyte ballooning than the ND-fed mouse strains, and no significant changes were observed between the two strains. The positive areas of Sirius Red staining in the livers of ND+CA-fed TSNO mice were significantly higher than in those of ND-fed mice (Figure 2C,D). Although ND-fed TSOD mice tended to show increased positive staining than ND-fed TSNO mice, no CA-induced changes similar to those observed in TSNO mice

were detected (Figure 2D). These findings indicated that the addition of CA to ND induced hepatic fibrosis, a key pathological feature of MASH, in TSNO mice, whereas such fibrotic changes were not evident in TSOD mice.

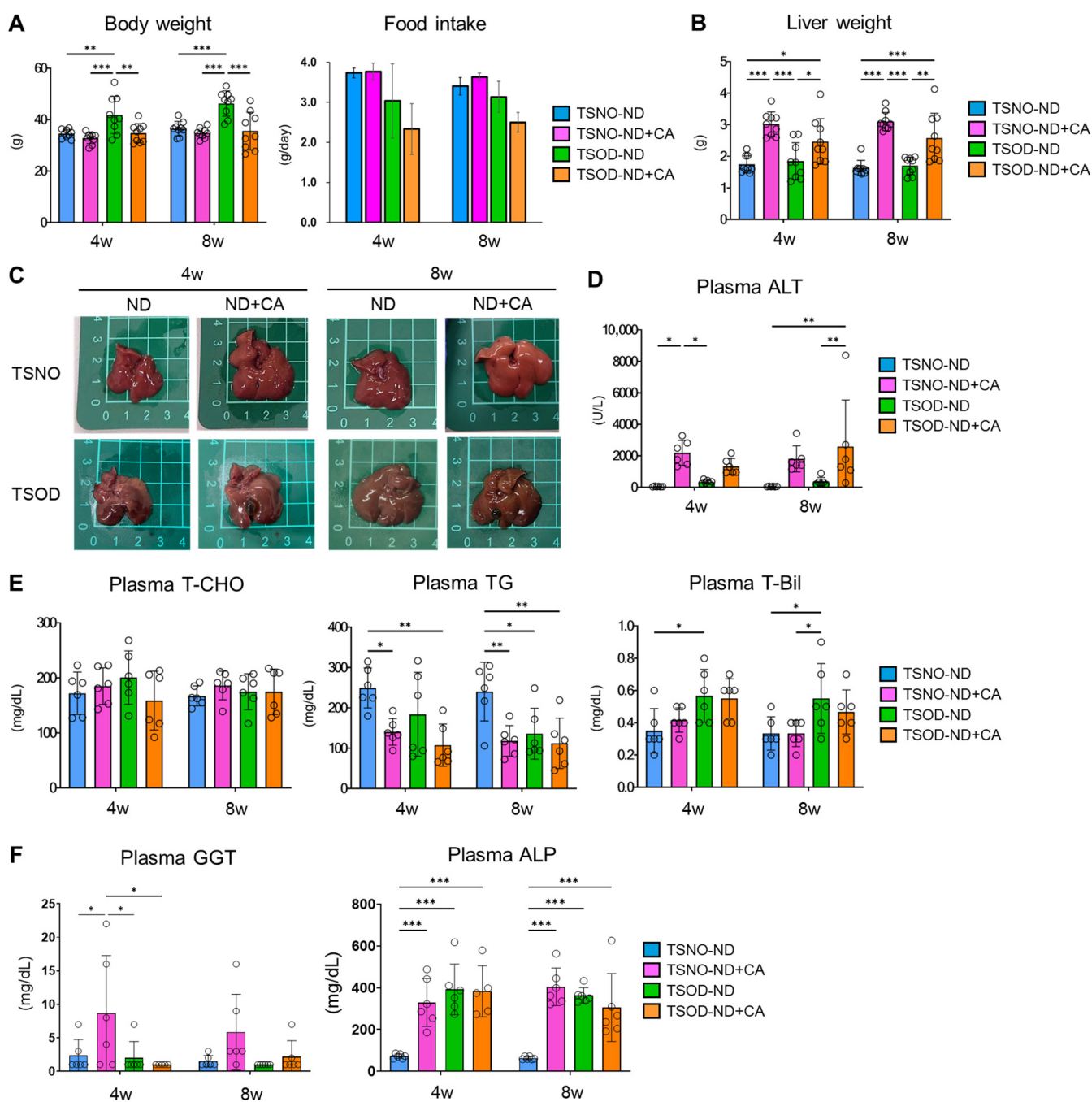


Figure 1. Effects of CA on liver size, hepatic injury, and lipid-related parameters. (A) Changes to body weight and food intake ($n = 9$). (B) Changes to liver weights ($n = 9$). (C) Representative liver images. (D) Plasma ALT concentrations ($n = 6$). (E) Plasma T-CHO, TG, and T-Bil ($n = 6$). (F) Plasma GGT and ALP ($n = 6$). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test or an unpaired Student's t -test. The value of n indicates the number of mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

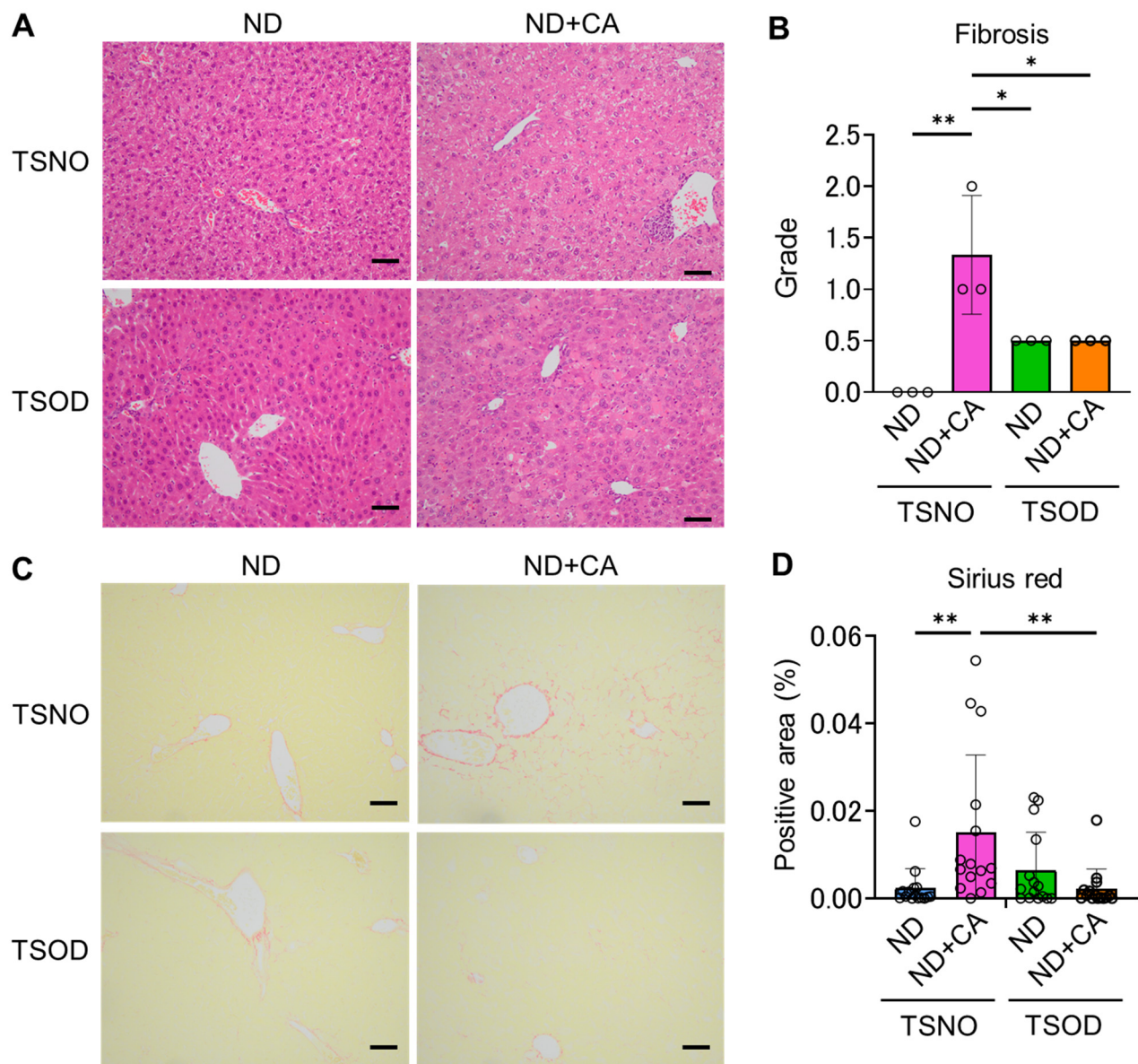


Figure 2. Effects of CA on liver histopathology. (A) H&E-stained sections. Scale bars, 100 μ m. (B) Liver fibrosis (0–4) was evaluated ($n = 3$). (C) Sirius Red-stained sections. Scale bars, 100 μ m. (D) For each group, images were obtained from five fields in three sections. Sirius Red-positive areas were quantified at 15 sites using ImageJ software. Statistical analyses were carried out using two-way ANOVA followed by Tukey's post hoc test or an unpaired Student's *t*-test, as indicated. The value of n represents the number of mice used for histological evaluation in each group. * $p < 0.05$, ** $p < 0.01$.

3.3. Dietary CA Upregulates the Expression Levels of Macrophage- and Fibrosis-Related Genes in the Liver of TSNO Mice

We examined the expression of inflammation-, macrophage-, and fibrosis-related genes in the liver by qRT-PCR (Figure 3). The expression levels of *Tnf* and *Il1b* were not significantly affected by CA supplementation in both strains. *Itgax* expression was elevated in ND+CA-fed TSNO mice than in ND-fed TSNO mice, with higher expression observed after 8 weeks than after 4 weeks of feeding. In TSOD mice, *Itgax* expression tended to increase with CA supplementation; however, the difference was not statistically significant between the diet groups. *Col1a1* expression was significantly upregulated in ND+CA-fed TSNO mice for 8 weeks than in ND-fed TSNO mice, whereas no significant change was observed in TSOD mice. A similar pattern was observed for *Timp1* expression, with a

significant increase in TSNO mice following CA supplementation at weeks 4 and 8, but not in TSOD mice. No significant differences in *Acta2* and *Tgfb1* expressions were observed between groups. *Vim*, a mesenchymal marker, showed a tendency to increase in response to CA supplementation in both strains; however, these changes did not reach statistical significance. These results indicate that CA supplementation of ND promoted CD11c⁺ cell infiltration and fibrosis in the livers of TSNO mice. These results indicate that CA supplementation of ND promoted CD11c⁺ cell infiltration and fibrosis in the livers of TSNO mice; however, CA alone is insufficient to strongly promote hepatic stellate cell activation in both strains.

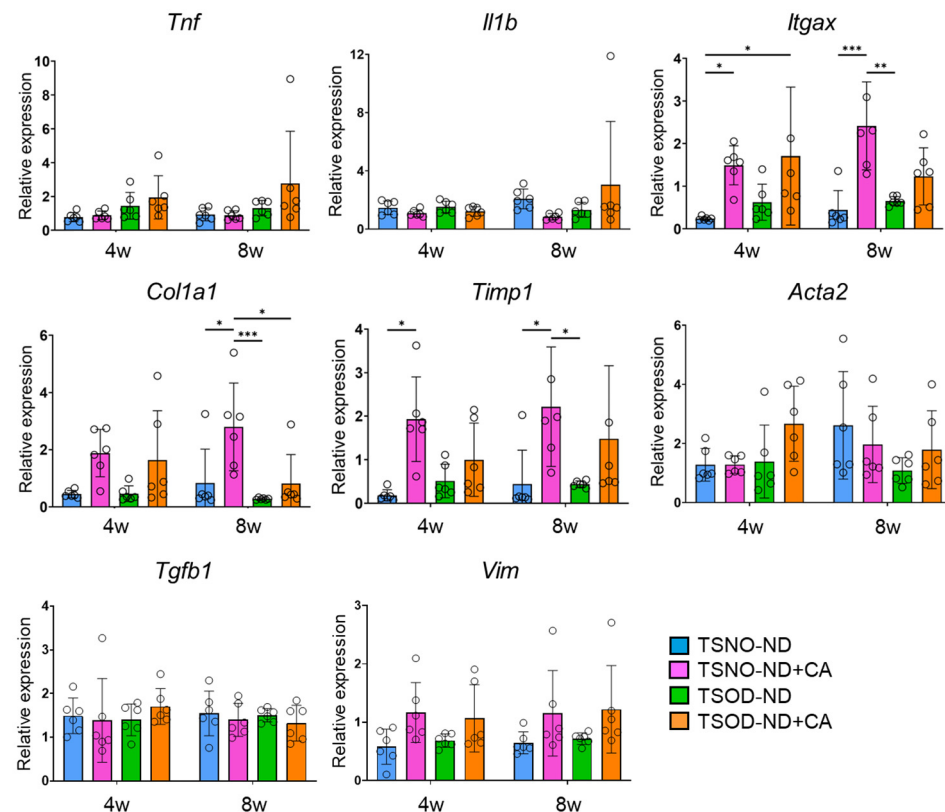


Figure 3. Effects of CA on hepatic expression of inflammatory and fibrotic markers. Hepatic mRNA levels of TNF- α (*Tnf*), IL-1 β (*Il1b*), CD11c (*Itgax*), collagen type 1 (*Col1a1*), TIMP-1 (*Timp1*), α SMA (*Acta2*), TGF- β (*Tgfb1*), and Vimentin (*Vim*) ($n = 6$). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test or an unpaired Student's *t*-test. The value of n denotes the number of mice for gene expression analysis in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4. Dietary CA Promotes Leukocyte Infiltration in the Livers of TSNO and TSOD Mice

Therefore, we investigated the immune cell infiltration in the liver using flow cytometry. Consistent with our previous findings [20], ND-fed TSOD mice exhibited a significantly higher proportion of CD45⁺ leukocytes in the liver than ND-fed TSNO mice (Figure 4A,B, left). Moreover, the proportion of CD45⁺ cells increased in both ND+CA-fed strains than in the ND-fed strains, with a time-dependent increase in TSNO mice (Figure 4A,B, left). Conversely, the proportion of CD45[−] cells significantly decreased in both strains with CA supplementation (Figure 4A,B, right). Similar to the proportions, the cell number of CD45⁺ cells increased in TSOD mice than in TSNO mice under ND feeding (Figure 4C, left). CA supplementation further increased the number of CD45⁺ cells compared with ND feeding in both strains, except in TSNO mice at 4 weeks and TSOD mice at 8 weeks (Figure 4C, left). In contrast, the number of CD45[−] cells showed considerable variability and no significant differences between the groups (Figure 4C, right). These findings suggested that CA sup-

plementation in the ND promoted hepatic leukocyte infiltration in TSNO and TSOD mice.

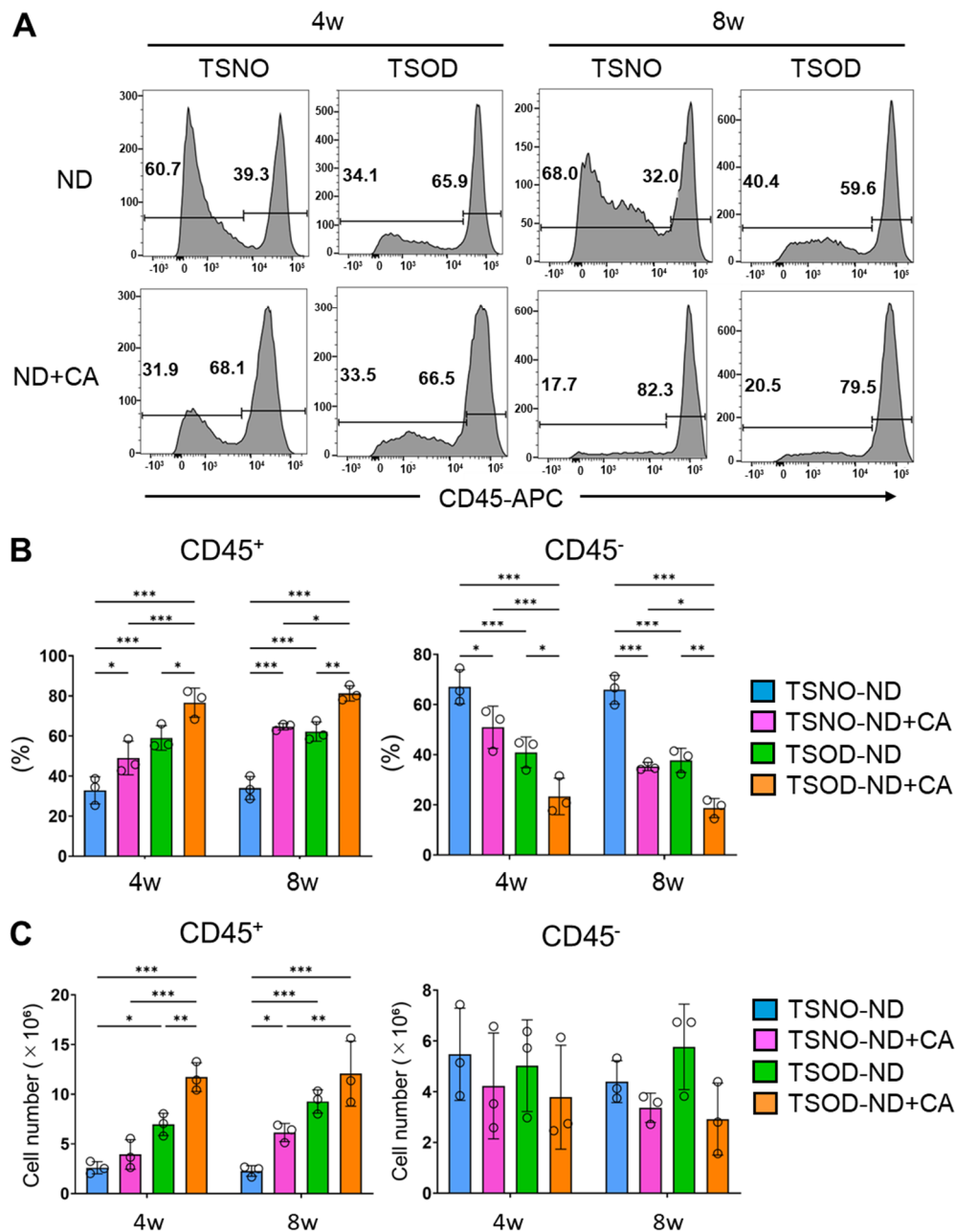


Figure 4. Effects of CA on hepatic accumulation of CD45⁺ leukocytes. **(A)** Flow cytometric profiles showing CD45 expression on non-parenchymal cells. **(B)** Percentages of live CD45⁺ (left) and CD45⁻ (right) non-parenchymal cells determined from the analysis shown in (A) ($n = 3$). **(C)** Absolute numbers of live CD45⁺ (left) and CD45⁻ (right) non-parenchymal cells calculated from data in (A). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test or an unpaired Student's *t*-test. The value of n represents the number of mice analyzed by flow cytometry in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5. Dietary CA Reduces KC Count and Promotes MdM Accumulation in the Liver

Therefore, we focused on macrophages among infiltrating leukocytes. Immunohistochemical staining for F4/80 showed a trend toward increased positive areas in TSNO mice following CA supplementation, although the difference was not significant (Figure S1A,B). F4/80-positive areas tended to decrease in ND+CA-fed TSOD mice than in ND-fed TSOD mice; however, this change was not statistically significant (Figure S1B).

In the ND-fed TSNO and TSOD mice, CD45⁺ cells in the liver were divided into two F4/80⁺ macrophage subsets: F4/80^{Hi}/CD11b^{Int} cells, corresponding to KCs, and F4/80^{Int}/CD11b^{Hi} cells, representing MdMs (Figure 5A) [20]. Similar to the observations under iHFC feeding [20], ND+CA feeding markedly reduced the proportion of KCs in TSNO mice compared to ND feeding (Figure 5B, left). In TSOD mice, the reduction in KC proportion was less pronounced than in TSNO mice, and no statistically significant differences were observed between the mice on different diets (Figure 5B, left). In contrast, the proportion of MdMs increased at 4 weeks in both strains under ND+CA feeding than under ND feeding (Figure 5B, right). In terms of cell number, the KC counts mirrored proportional changes, showing a greater reduction in TSNO mice than in TSOD mice upon CA supplementation, although these differences were not statistically significant (Figure 5C, left). Notably, MdM numbers were significantly increased in TSOD mice fed ND+CA for 4 weeks than those fed ND (Figure 5C, right). These findings suggested that dietary CA reduced KC levels and promoted MdM accumulation in the liver.

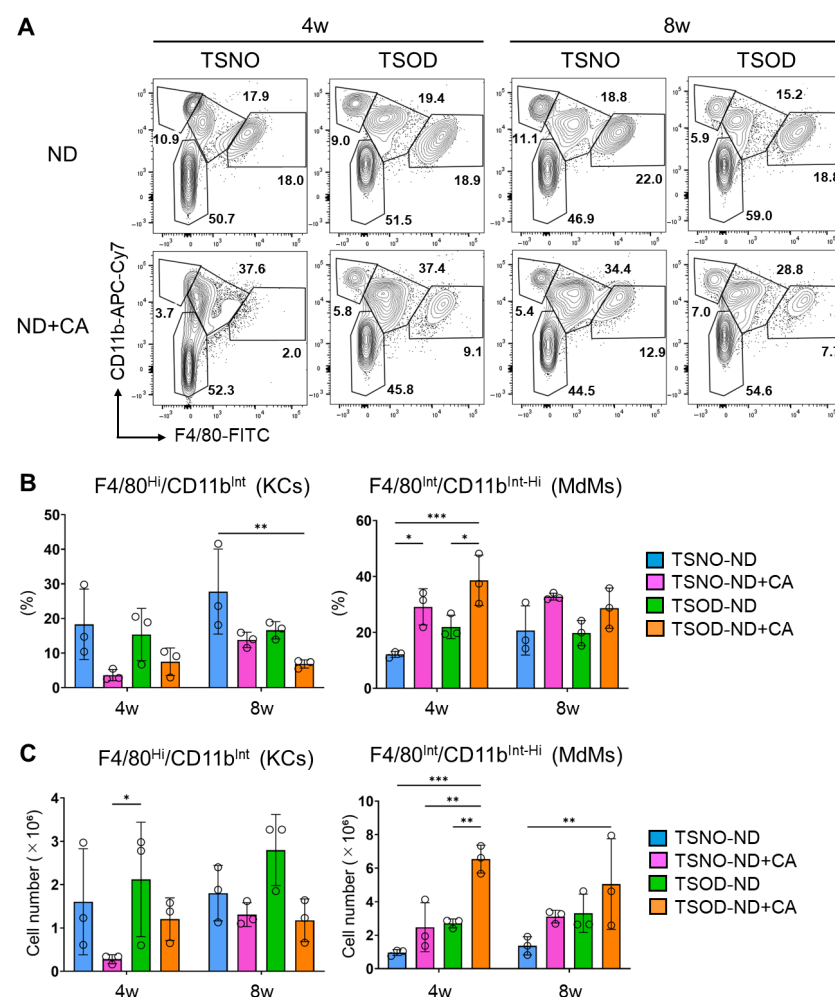


Figure 5. Effects of CA on KCs and MdMs populations in the livers. (A) Flow cytometric plots showing F4/80 and CD11b expression on CD45⁺ non-parenchymal cells including KCs. (B) Percentages of F4/80^{Hi}/CD11b^{Int} KCs (left) and F4/80^{Int}/CD11b^{Int-Hi} MdM (right) ($n = 3$). (C) Absolute numbers of F4/80^{Hi}/CD11b^{Int} KCs (left) and F4/80^{Int}/CD11b^{Int-Hi} MdMs (right) ($n = 3$). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test or an unpaired Student's t -test. The value of n denotes the number of mice subjected to flow cytometric analysis in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6. CA Supplementation to ND Induces CD11c⁺/Ly6C[−] and CD11c[−]/Ly6C⁺ Mdm Subset Accumulation

MdMs were classified into three distinct subsets according to the CD11c and Ly6C expression levels in iHFC diet-fed TSNO mice [19]. The proportions of CD11c⁺/Ly6C[−] and CD11c[−]/Ly6C⁺ cells in the liver of ND-fed TSNO mice were minimal (Figure 6A) [19]. In contrast, ND-fed TSOD mice exhibited a higher proportion of CD11c⁺/Ly6C[−] cells and markedly greater proportion of CD11c[−]/Ly6C⁺ cells than ND-fed TSNO mice (Figure 6A) [20]. In TSNO mice, CA supplementation significantly increased the proportions of both subsets at 4 and 8 weeks than ND feeding (Figure 6A,B). Only the proportion of CD11c⁺/Ly6C[−] cells significantly increased in ND+CA-fed TSOD mice at 4 weeks (Figure 6B). Although CA supplementation tended to increase the number of both subsets in TSNO mice, the differences were not significant compared to ND (Figure 6C). In TSOD mice, significant differences in cell numbers between diets were observed for both subsets, except for CD11c[−]/Ly6C⁺ cells at 8 weeks (Figure 6C). Collectively, these results indicate that ND supplementation with CA induces changes in both Mdm subsets in TSOD and TSNO mice, mirroring those observed with iHFC feeding.

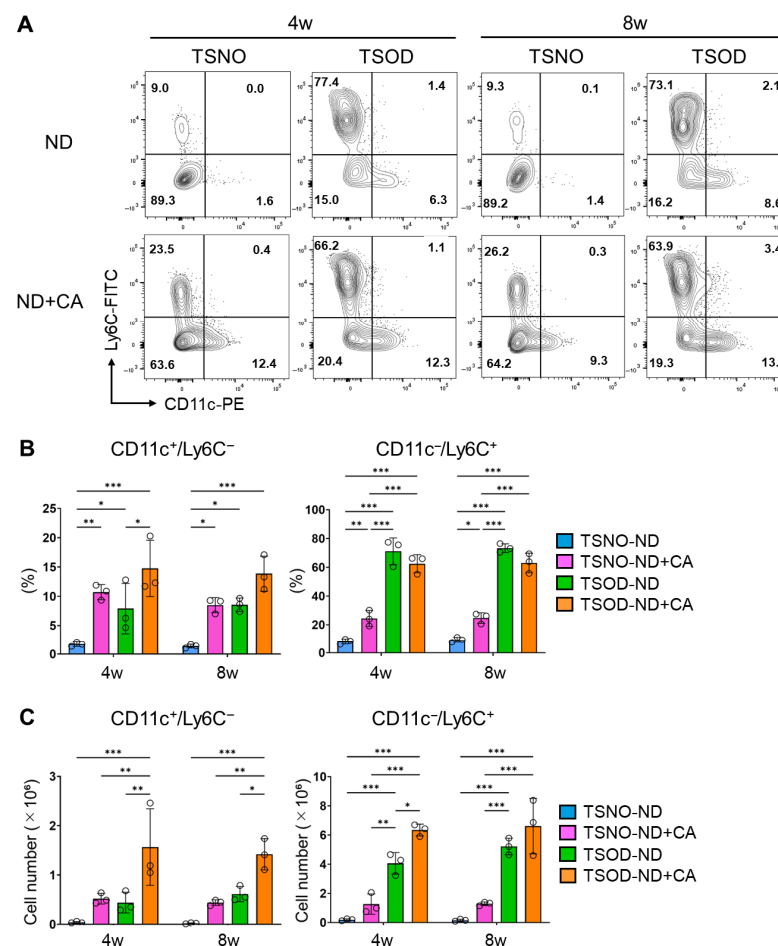


Figure 6. Effects of CA on CD11c⁺/Ly6C[−] and CD11c[−]/Ly6C⁺ MDMs. **(A)** Flow cytometric profiles showing CD11c and Ly6C expression on F4/80⁺ recruited macrophages. **(B)** Percentages of CD11c⁺/Ly6C[−] (left) and CD11c[−]/Ly6C⁺ (right) ($n = 3$). **(C)** Absolute numbers of CD11c⁺/Ly6C[−] (left) and CD11c[−]/Ly6C⁺ (right) ($n = 3$). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test or an unpaired Student's t -test. The value of n represents the number of mice for flow cytometric analysis in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.7. CA Is Essential for the iHFC-Induced Increase in Intestinal Abundance of *A. muciniphila*

α -diversity and principal component analysis (PCA) of gut microbiota composition based on 16S rRNA sequencing revealed distinct clustering between ND- or ND+CA-fed TSOD and TSNO mice (Figure 7A,B). CA supplementation significantly shifted the α -diversity in TSOD mice (Figure 7A). Compositional differences were evident among ND-fed strains (Figure 7B). Notably, CA supplementation markedly shifted the microbial profiles of both strains, with a clear separation between the ND- and ND+CA-fed mice (Figure 7B). ND-fed TSOD mice exhibited a higher proportion of Gram-positive bacteria than ND-fed TSNO mice, whereas CA supplementation significantly increased the abundance of Gram-negative bacteria in TSOD mice (Figure 7C,D). At the phylum level, the ND-fed TSOD mice exhibited lower levels of Bacteroidota and higher levels of Firmicutes than the ND-fed TSNO mice (Figure 7E,F). CA supplementation significantly decreased Firmicutes and increased Verrucomicrobiota in the TSOD mice (Figure 7E,F). Log-ratio analysis of bacterial family level abundance demonstrated significant alterations in the microbial composition after CA supplementation (Figure 7G). The ND-fed TSOD mice exhibited higher relative abundances of Bifidobacteriaceae, Erysipelotrichaceae, Clostridiaceae, and Peptostreptococcaceae than the ND-fed TSNO mice (Figure 7G). Among these, Bifidobacteriaceae and Erysipelotrichaceae were significantly reduced in the TSOD mice following CA supplementation (Figure 7G). In addition, the abundance of Lactobacillaceae decreased in TSOD mice following CA supplementation (Figure 7G). Furthermore, CA feeding markedly increased the relative abundance of Bacteroidaceae, Tannerellaceae, and Akkermansiaceae, a family within the Verrucomicrobiota in TSOD mice than ND feeding (Figure 7G). These alterations were comparatively modest in TSNO mice (Figure 7G). The abundance of *Akkermansia muciniphila* in fecal samples was quantified using real-time PCR (Figure 7H). Under ND conditions, *A. muciniphila* levels were comparable between TSNO and TSOD mice (Figure 7H). However, CA supplementation significantly increased *A. muciniphila* abundance in TSOD mice than in ND-fed TSOD mice (Figure 7H). In contrast, CA had little effect on *A. muciniphila* levels in the TSNO mice (Figure 7H). These findings suggest that the impact of CA-supplemented normal diet on gut microbiota composition is greater in TSOD mice than in TSNO mice. Furthermore, CA-induced alterations in the gut microbiota appeared to influence obesity and T2DM pathophysiology in TSOD mice but did not appear to contribute to the changes in liver fibrosis observed in TSNO mice.

3.8. Fecal BA Profiles Reveal Differential Responses to CA Supplementation in TSOD and TSNO Mice

Fecal BA composition was analyzed to assess the effects of dietary CA supplementation on intestinal BA metabolism (Figure 8). CA supplementation markedly increased the proportions of CA, taurocholic acid (TCA), and taurodeoxycholic acid (TDCA) in both strains, whereas the proportion of muricholic acid (MCAs) decreased (Figure 8A). This shift indicates enhanced intestinal conversion toward CA-derived secondary BAs after dietary CA exposure. Quantitative analysis revealed that total unconjugated BA concentration increased with CA supplementation in both strains, with TSNO mice exhibiting higher overall levels than TSOD mice (Figure 8B, left). In contrast, CA feeding increased conjugated BA concentrations, predominantly in TSNO mice (Figure 8B, right), suggesting active enterohepatic recirculation. Individual analyses of unconjugated BAs showed that fecal levels of CA and DCA were significantly increased in TSNO mice receiving CA supplementation (Figure 8C). Particularly, lithocholic acid (LCA) and ω MCA levels were significantly decreased in the ND+CA-fed TSNO mice compared with in ND-fed controls (Figure 8C). In contrast, MCAs levels were unchanged in TSOD mice (Figure 8C), indicating strain-dependent differences in microbial BA metabolism. Among the conjugated BAs

(Figure 8D), TCA, TDCA, and taumuricholic acid (TMCA) levels increased in TSNO mice, whereas TDCA levels were increased in TSOD mice. However, none of these differences were statistically significant from the levels in ND-fed controls (Figure 8D).

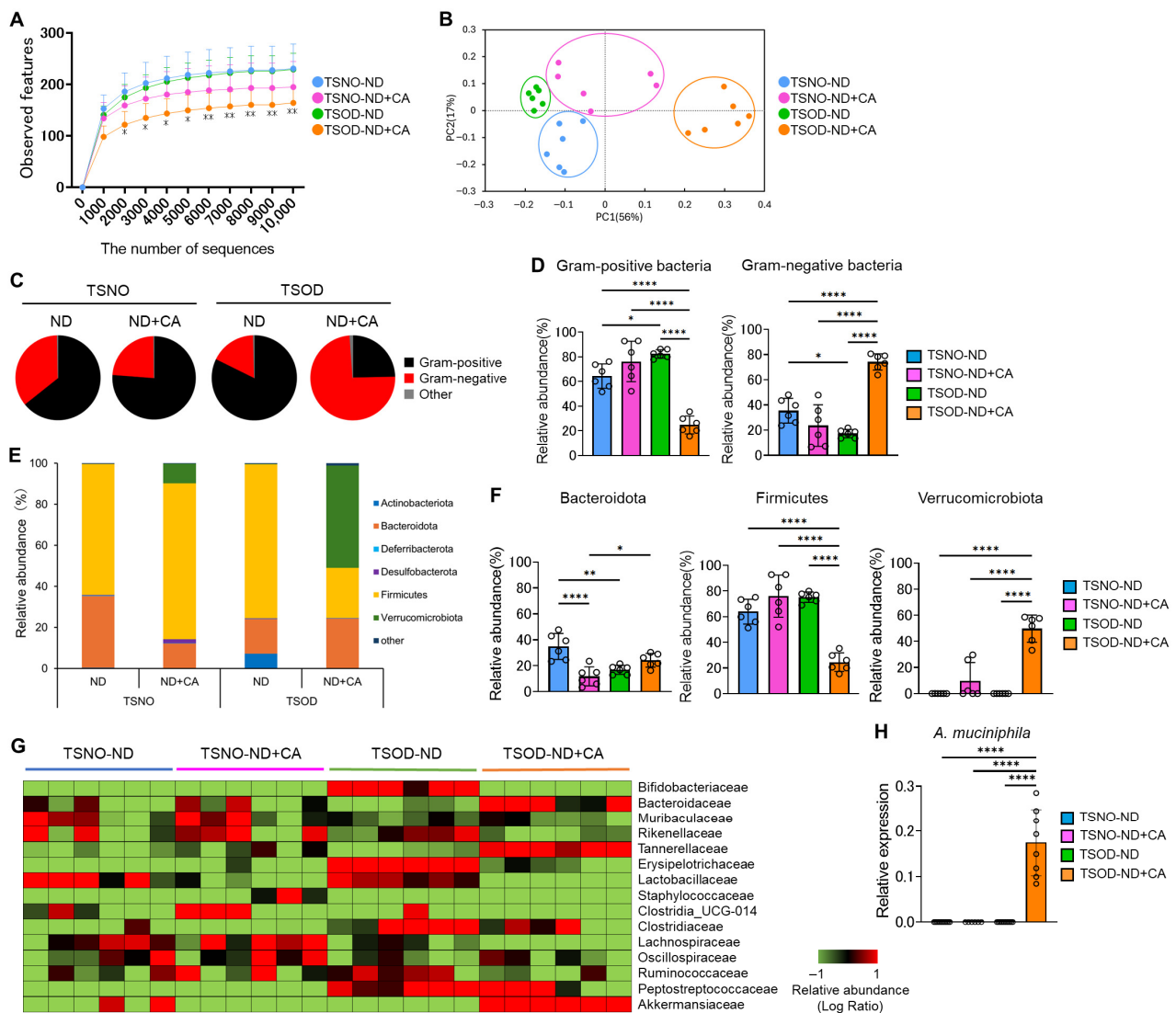


Figure 7. Effects of CA on gut microbiota profiles. Fecal samples were obtained after 4 weeks of dietary intervention. (A) Influence of CA on the α -diversity (Faith-PD and Observed features) of intestinal bacteria ($n = 6$). * $p < 0.05$ for TSOD-ND vs. TSOD-ND+CA, ** $p < 0.01$ for TSOD-ND vs. TSOD-ND+CA. (B) Principal coordinate analysis of β -diversity based on Bray–Curtis distances metrics ($n = 6$). (C) Proportions of Gram-positive, Gram-negative, and other bacterial groups ($n = 6$). (D) Comparison of gram-positive and gram-negative bacterial percentages ($n = 6$). (E) Relative abundance of bacterial phyla ($n = 6$); only taxa with a mean abundance greater than 1% are shown. (F) Relative abundance of Bacteroidota, Firmicutes, and Verrucomicrobia ($n = 6$). (G) Representation of relative abundance of bacterial families ($n = 6$); taxa with a mean abundance above 1% are included. (H) Relative levels of *Akkermansia muciniphila* ($n = 6$ –12). Statistical analyses were carried out using an unpaired Student's t -test or two-way ANOVA followed by Tukey's post hoc test or an unpaired Student's t -test, as appropriate. The value of n represents the number of mice per group for panels (A–G) or the number of fecal samples per group for panel (H) used in microbiota analyses. (D,F,H) * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

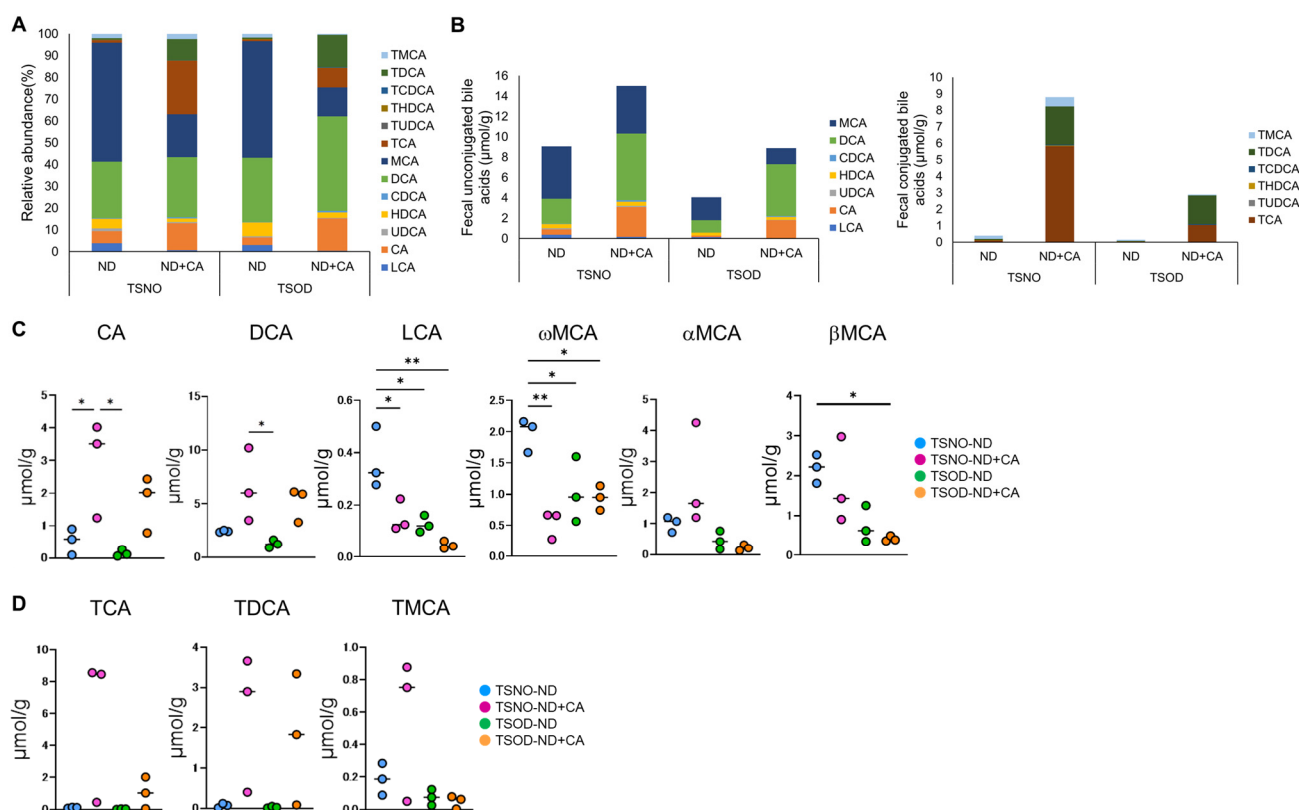


Figure 8. Effects of CA on fecal BA composition. (A) Overall BA profiles in fecal samples after 4 weeks of feeding ($n = 3$). (B) Levels of unconjugated (left) and conjugated BAs (right) ($n = 3$). (C,D) Alterations in individual unconjugated (C) and conjugated (D) BAs ($n = 3$). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test or an unpaired Student's t -test. The value of n denotes the number of mice per group included in the bile acid analysis. * $p < 0.05$, ** $p < 0.01$.

Taken together, these findings demonstrate that dietary CA alters the fecal BA composition in a strain-dependent manner. Compared to TSOD mice, TSNO mice exhibited broader increases in both primary and secondary BAs, including the conjugated forms. These differences likely reflect distinct host–microbe interactions and BA transformation capacities between the two strains.

4. Discussion

In this study, we examined the effects of dietary CA supplementation on hepatic inflammation, fibrosis, and the gut microbiota in ND-fed TSNO and TSOD mice, which exhibited distinct metabolic and immunological characteristics. The results demonstrated that CA induced hepatomegaly and liver injury in both strains; however, fibrotic changes were prominent in TSNO mice. In contrast, CA supplementation in TSOD mice significantly altered the gut microbiota composition and fecal BA profiles, particularly those enriched *A. muciniphila*. These findings suggest that dietary CA exerts strain-dependent effects on the gut–liver axis and may differentially influence MASH-related pathologies. Furthermore, these results correlate well with our previous findings from TSNO and TSOD mice fed an iHFC diet without CA [21,22], supporting the consistent role of CA in modulating MASH progression.

4.1. Hepatic Effects of Dietary CA

CA supplementation caused hepatomegaly and increased plasma ALT levels in both the TSNO and TSOD mice, indicating hepatocellular injury. Histological and molecular analyses further revealed that CA promoted hepatic fibrosis in TSNO mice, as evidenced

by increased Sirius red-positive areas and upregulated *Col1a1* and *Timp1* expression. These findings are in agreement with earlier studies demonstrating that cholate-containing diets can accelerate liver injury and fibrogenesis in rodent models [10,12,18]. In contrast, TSOD mice, which exhibit obesity and insulin resistance, show a limited fibrotic response to CA [20]. The relatively modest hepatic changes observed in TSOD mice may reflect differences in BA handling or adaptive mechanisms related to their metabolic backgrounds.

To further characterize the strain-dependent hepatic response to dietary CA, we examined plasma markers associated with BA-related hepatic and biliary stress. CA supplementation induced significant increases in both GGT and ALP levels in TSNO mice, whereas no such increases were observed in TSOD mice. Notably, TSOD mice exhibited elevated basal ALP levels under ND conditions compared with TSNO mice, and CA supplementation did not further augment ALP levels. GGT and ALP are commonly used markers of BA-related hepatic and biliary stress. The increases observed in TSNO mice likely reflect an acute biochemical response to excess dietary CA, suggesting that BA overload induces detectable hepatic and biliary stress in metabolically normal mice. In contrast, the lack of GGT and ALP elevation in TSOD mice indicates an attenuated response to CA supplementation. The elevated basal ALP levels in ND-fed TSOD mice suggest the presence of pre-existing chronic hepatic or biliary stress associated with obesity, insulin resistance, and metabolic dysfunction. In this context, additional CA exposure may fail to elicit further increases in circulating GGT or ALP, possibly due to a ceiling effect or impaired adaptive responsiveness of BA metabolism and signaling pathways. Such blunted biochemical responses are consistent with altered BA homeostasis reported in metabolically dysregulated states [25]. Taken together, these findings indicate that the metabolic background critically influences hepatic and biliary responses to dietary CA.

4.2. Alterations in Hepatic Macrophage Populations

Flow cytometric analysis demonstrated that CA supplementation reduced the KC count, while increasing MdMs in both strains. Among the MdM subsets, the proportion of CD11c⁺/Ly6C[−] cells markedly increased in CA-fed TSNO mice. This subset has been previously associated with hCLS and fibrotic activity in MASH models [7,19]. The concurrently increased *Itgax* expression supports CD11c⁺ macrophage expansion. The loss of KCs may result from cytotoxic effects of BAs or inflammation-induced depletion, as previously reported [26]. Taken together, these observations suggest that dietary CA modifies macrophage dynamics and promotes a profibrotic milieu, particularly in TSNO mice. However, further mechanistic studies should aim to determine whether these changes are a direct consequence of CA signaling or secondary to hepatocellular stress.

4.3. Effects of CA on Gut Microbiota and BA Metabolism

CA supplementation markedly altered the gut microbial composition, particularly in TSOD mice. Consistent with earlier findings that BAs regulate microbial ecology through antimicrobial activity and signaling via FXR and TGR5 [10,13,14], CA reduced the abundance of Firmicutes and increased the abundance of Verrucomicrobiota, including *A. muciniphila*. Moreover, these findings are in line with our earlier observation that *A. muciniphila* increased in TSOD mice fed an iHFC diet, whereas this increase was abolished when CA was removed from the diet [22]. The increased *A. muciniphila* abundance observed in the CA-fed TSOD mice is noteworthy because this bacterium improves metabolic homeostasis and intestinal barrier integrity in both mice and humans [15,16]. Such changes in the microbiota may mitigate hepatic inflammation in TSOD mice, potentially explaining the limited fibrogenic response to CA exposure. However, because microbiota-targeted interventions such as fecal transplantation or antibiotic treatment were not performed in this

study, the observed relationships between gut microbiota alterations and hepatic outcomes should be interpreted as correlative rather than causal. Furthermore, differences in fecal BA composition between strains indicate that the host metabolic background strongly influences BA metabolism and microbial adaptation, consistent with recent evidence of bidirectional crosstalk between BAs and gut microbiota [13,14,27].

4.4. Integration of Hepatic and Intestinal Responses

The contrasting responses observed in the TSNO and TSOD mice suggest that the pathological effects of dietary CA depend on the metabolic state of the host. CA primarily enhances hepatic macrophage activation and fibrogenesis in TSNO mice and predominantly affects the intestinal environment and BA metabolism in TSOD mice. These strain-dependent outcomes highlight the complex interplay between metabolic dysfunction, BA signaling, and immune regulation in MASH progression [4,10,28]. Further investigation of the FXR–TGR5 axis and downstream cytokine networks may help clarify how dietary CA differentially influences the gut–liver axis in distinct metabolic contexts.

4.5. Limitations and Perspectives

This study had several limitations. First, the experimental period was relatively short and the long-term effects of CA on liver fibrosis and metabolic homeostasis remain unclear. Second, although flow cytometry identified changes in macrophage subsets, additional transcriptomic or functional analyses were necessary to elucidate the mechanisms underlying CA-induced macrophage polarization. Third, although an association between CA supplementation and *A. muciniphila* enrichment was evident, causality could not be confirmed in the absence of microbiota manipulation. Fourth, hepatic fibrosis was evaluated mainly by histological quantification using Sirius Red staining and by mRNA expression analysis of fibrotic markers, such as *Col1a1* and *Acta2*, without parallel assessment at the protein level. Although Sirius Red-positive area measurement is a well-established method for assessing collagen deposition, gene expression does not necessarily reflect protein expression; therefore our results suggest a pro-fibrotic tendency but do not provide definitive evidence of established fibrosis. In future studies, we would like to strengthen the molecular and histological support for fibrosis by incorporating protein-based analyses (e.g., immunoblotting or immunostaining for collagen I or α -SMA), as well as additional staining approaches such as Masson's trichrome staining, Gomori's trichrome staining, aniline staining, or protein-specific immunohistochemistry. Despite these limitations, our findings provide a foundation for future studies aimed at elucidating the effect of dietary CA on hepatic immune responses and microbial dynamics in MASH.

4.6. Conclusions

In summary, dietary CA supplementation induced hepatocellular injury and fibrosis in TSNO mice, but altered the gut microbiota and BA composition in TSOD mice. These results suggested that the effect of CA on MASH pathogenesis strongly depends on the host's metabolic background. Further mechanistic studies integrating BA signaling, immune cell dynamics, and microbiota metabolism are essential to clarify the multifaceted roles of dietary CA in metabolic liver diseases.

Supplementary Materials: The following supporting information can be downloaded from: <https://www.mdpi.com/article/10.3390/biomedicines14020442/s1>, Figure S1: Accumulation of F4/80⁺ macrophages in livers of TSNO and TSOD mice; Table S1: Antibodies for flow cytometry; Table S2: Primers for RT-qPCR.

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K.T. and Y.F.; Writing—original draft preparation: Y.N.; Writing—review and editing: Y.N. and T.A.; Supervision, Y.N.; Project administration: Y.N.; Funding acquisition: Y.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Animal experiments were carried out in compliance with the ARRIVE guidelines, covering study design, statistical analysis, experimental procedures, and the care and housing of animals, together with the guidelines for the Proper Conduct of Animal Experiments established by the Science Council of Japan. The Ethics Committee for Animal Experiments at Toyama Prefectural University approved the animal experimental protocols (R1-3, R4-1, and R5-21).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting the results of this study can be obtained from the corresponding author upon request.

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Abbreviations

7-AAD	7-amino-actinomycin D
ALP	alkaline phosphatase
ALT	alanine aminotransferase
BA	bile acid
CA	cholic acid
DCA	deoxycholic acid
FXR	farnesoid X receptor
GGT	γ -glutamyltransferase
hCLS	hepatic crown-like structure
H&E	hematoxylin and eosin
KC	Kupffer cell
LCA	lithocholic acid
MASH	metabolic dysfunction-associated steatohepatitis
MCA	muricholic acid
MdM	monocyte-derived macrophage
NAS	NAFLD activity score
ND	normal diet
PCA	principal component analysis
T2DM	type 2 diabetes mellitus
T-Bil	total bilirubin
TCA	taurocholic acid
T-CHO	total cholesterol
TDCA	taurodeoxycholic acid
TG	triglyceride
TMCA	taumuricholic acid
TSNO	Tsumura–Suzuki non-obese
TSOD	Tsumura–Suzuki obese diabetes

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