


Hepatic Adenosine Triphosphate Reduction Through the Short-Chain Fatty Acids–Peroxisome Proliferator-Activated Receptor γ –Uncoupling Protein 2 Axis Alleviates Immune-Mediated Acute Hepatitis in Inulin-Supplemented Mice

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How liver tolerance is disrupted in immune-mediated liver injury is currently unclear. There is also insufficient information available regarding susceptibility, precipitation, escalation, and perpetuation of autoimmune hepatitis. To explore how dietary fiber influences hepatic damage, we applied the concanavalin A (ConA)-induced acute immune-mediated liver injury model in mice fed a diet supplemented with 6.8% inulin, a water-soluble fermentable fiber. Twelve hours after ConA administration, inulin-supplemented diet-fed mice demonstrated significantly alleviated hepatic damage histologically and serologically, with down-regulation of hepatic interferon- γ and tumor necrosis factor and reduced myeloperoxidase (MPO)-producing neutrophil infiltration. Preconditioning with an inulin-supplemented diet for 2 weeks significantly reduced hepatic adenosine triphosphate (ATP) content; suramin, a purinergic P2 receptor antagonist, abolished the protective effect. Of note, the portal plasma derived from mice fed the inulin-supplemented diet significantly alleviated ConA-induced immune-mediated liver injury. Mechanistically, increased portal short-chain fatty acid (SCFA) levels, such as those of acetate and butyrate, by inulin supplementation leads to up-regulation of hepatic γ -type peroxisome proliferator-activated receptor (*Pparg*) and uncoupling protein 2 (*Ucp2*), which uncouples mitochondrial ATP synthesis downstream of PPAR γ . *Pparg* down-regulating small interfering RNA cancelled the protective effect of inulin supplementation against MPO-producing neutrophil infiltration and the subsequent immune-mediated liver injury, suggesting that the SCFA–PPAR γ –UCP2 axis plays a key role in the protective effect by inulin supplementation. Moreover, significant changes in the gut microbiota, including increased operational taxonomic units in genera *Akkermansia* and *Allobaculum*, also characterized the protective effect of the inulin-supplemented diet. **Conclusion:** There is a possible unraveled etiopathophysiological link between the maintenance of liver tolerance and dietary fiber. The SCFA–PPAR γ –UCP2 axis may provide therapeutic targets for immune-mediated liver injury in the future. (*Hepatology Communications* 2021;5:1555-1570).

Liver tolerance, the physiological predisposition toward immune unresponsiveness, is an important adaptive response that limits exaggerated tissue damage.⁽¹⁾ However, immune tolerance in the liver can be disturbed, resulting in robust, hepatic, immunologic reactions against pathogens

Abbreviations: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; APC, allophycocyanin; ATP, adenosine triphosphate; BV, Brilliant Violet; CD, cluster of differentiation; ConA, concanavalin A; Cont, control; Cy7, cyanine 7 dye; FITC, fluorescein isothiocyanate; Fsp27, fat-specific protein 27; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPR, G-protein coupled receptor; H&E, hematoxylin and eosin; IFN, interferon; IL, interleukin; Inu, inulin; KO, knockout; Ly6, lymphocyte antigen 6; MPO, myeloperoxidase; mRNA, messenger RNA; NK, natural killer; NS, not significant; OTU, operational taxonomic unit; PCR, polymerase chain reaction; PE, phycoerythrin; PPAR, peroxisome proliferator-activated receptor; RAG2, recombination activating gene 2 locus; rRNA, ribosomal RNA; SCFA, short-chain fatty acid; siRNA, small interfering RNA; TNF, tumor necrosis factor; Tregs, regulatory T cells; UCP2, uncoupling protein 2; WT, wild type.

(such as hepatitis A or B), toxins, or sometimes self-antigens, leading to autoimmune hepatitis (AIH).⁽²⁾ All such etiologies may further cause massive cell death in the liver, a fatal syndrome called acute liver failure in which liver transplantation is the only established treatment option.⁽³⁾

Various pathogenic mechanisms have been implicated in AIH; however, none perfectly explain the susceptibility, precipitation, escalation, and perpetuation of AIH.⁽⁴⁾ A gradual increase in the incidence of AIH has been reported in Denmark.⁽⁵⁾ In a recent nationwide survey in Japan, the incidence of AIH with acute presentation has increased in this decade.⁽⁶⁾ A European survey demonstrated that the number of liver transplantations (LTs) for AIH remained unchanged from 1988 to 2009, while LTs for primary biliary cholangitis, another disease entity of autoimmune origin, has declined despite the rising prevalence of this disease.⁽⁷⁾ Better clinical management for patients with AIH remains an unmet need.⁽⁸⁾ Environmental factors, such as socioeconomic status and sanitation, have been the focus for

the maintenance of immune tolerance in allergic and autoimmune diseases (the “hygiene hypothesis”),⁽⁹⁾ and gut microbiota have been highlighted in the pathogenesis of some of them.⁽¹⁰⁾ To date, there have been only two studies regarding the gut microbiota in AIH^(11,12); however, the role of gut microbiota in AIH pathogenesis is currently unknown.

Since the publication of “Burkitt’s hypothesis” in 1969,⁽¹³⁾ large-scale epidemiological studies have reported that dietary fiber deficiency increases the risk of colon, liver, and breast cancer and mortality from cardiovascular, infectious, and respiratory diseases as well as from diabetes, noncardiovascular diseases, and cancer.⁽¹⁴⁾ Most studies regarding lifestyle-associated hepatic diseases have focused on high calorie, high fat, or high cholesterol; only some studies have demonstrated that dietary fiber intake improves nonalcoholic steatohepatitis (NASH) and decreases the occurrence of hepatocellular carcinoma.^(15,16) How dietary fiber along with subsequent microbiomic or metabolomic changes affect liver tolerance remains ill defined. Moreover, a decreasing trend in dietary fiber intake in

Received February 8, 2021; accepted April 24, 2021.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1742/supinfo.

Supported in part by the Keio University Medical Fund N.N.).

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DOI 10.1002/hep4.1742

Potential conflict of interest: Nothing to report.

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Japan is anticipated.⁽¹⁷⁾ Its relationship to the increasing trend in acute-onset AIH is also of great interest. Inulin, one of the major nondigestible oligosaccharides,⁽¹⁸⁾ has prebiotic effects through the production of short-chain fatty acids (SCFAs) after fermentation in the intestines.⁽¹⁹⁾ Acetic acid, propionic acid, and butyric acid, three typical SCFAs, are usually emphasized for their contribution to gluconeogenesis and lipogenesis in the context of NASH⁽²⁰⁾; however, their roles in immune-mediated liver injury remain unclear.

Since the first report of the intravenous injection of concanavalin A (ConA) (a well-known lectin-type T-cell mitogen) to establish a murine T-cell-mediated acute liver injury,⁽²¹⁾ it has been widely applied to investigate immune-mediated liver injury. ConA hepatitis also resembles human AIH because interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) are critical for their pathogenesis.^(22,23) In the current study, we investigated the effect of inulin supplementation per os in a ConA-induced model of acute immune-mediated liver injury in mice. We demonstrated that the reduction of intrahepatic adenosine triphosphate (ATP), which limited neutrophil infiltration through purinergic P2 receptors, by the SCFA-peroxisome proliferator-activated receptor γ (PPAR γ)-uncoupling protein 2 (UCP2) axis, alleviated immune-mediated liver injury. Our findings suggest a causal relationship between dietary fiber deficiency and a predisposition to disrupting liver tolerance and to suffering tissue damage in immune-mediated liver injury.

Materials and Methods

MICE AND DIET PREPARATION

Wild-type (WT) C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). G-protein coupled receptors (*GPR*) 41 and *GPR*43 knockout (KO) mice were kindly provided by Dr. Ikuo Kimura (Kyoto University, Kyoto, Japan). Interleukin (*IL*)10 KO mice were purchased from Jackson Laboratories (Bar Harbor, ME). Recombination activating gene 2 locus (*RAG2*) KO mice were purchased from Central Laboratories for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Keio University School of Medicine. Experiments were performed with age- and sex-matched mice at

6-12 weeks of age. All experiments were approved by the animal ethics committee of Keio University, Tokyo, Japan, and performed according to the guidelines. Mice were fed with free access to either control diet (Cont-diet; AIN93-G) or inulin diet (Inu-diet; 6.8% inulin-supplemented diet replacing 5.0% cellulose and 1.8% cornstarch) (Supporting Fig. S1A). The proportion of inulin in the Inu-diet was similar to what has been reported.⁽²⁴⁾ All experiments were conducted in conditions without artificial fasting.

ConA-INDUCED ACUTE LIVER INJURY MODEL AND REAGENTS

ConA (type IV) was purchased from Sigma-Aldrich (St Louis, MO). Phosphate-buffered saline or ConA (20 mg/kg) was intravenously administered to the tail vein of euthanized mice 12 hours before tissue harvest. In some experiments, *RAG2* KO mice received ConA followed by intraperitoneal treatment with recombinant IFN- γ (rIFN- γ ; 500 ng) and recombinant TNF (rTNF; 500 ng) as reported.⁽²⁵⁾ After being fed a Cont- or Inu-diet for 2 weeks, some mice were coinjected with ConA and the purinergic P2 receptor antagonist suramin (Sigma-Aldrich; 200 μ g/g body weight dissolved in pure water, intraperitoneally). Some Cont- or Inu-diet-fed mice were intravenously administered plasma of portal vein blood (200 μ L/mouse) derived from ConA-treated mice 2 hours before ConA administration, as described.⁽²⁶⁾

BIOCHEMICAL AND HISTOLOGIC ASSESSMENT

After the mice were euthanized, peripheral or portal vein blood plasma or serum was centrifuged at 1,500g for 10 minutes and stored at -80°C . Serum alanine aminotransferase (ALT) levels were determined using a DRI-CHEM 3500i Analyzer (Fuji Film, Tokyo, Japan). Livers were fixed in 10% buffered formalin and embedded in paraffin. The prepared sections were stained with hematoxylin and eosin (H&E) and examined.

PREPARATION OF LIVER MONONUCLEAR CELLS

Liver mononuclear cells were separated from the liver as described.⁽²⁷⁾ (See Supporting Methods for details.)

16S RIBOSOMAL RNA METAGENOMICS ANALYSIS

Fecal samples were collected from mice housed individually in separate cages after being fed the Cont- or Inu-diet for 2 weeks. Samples were immediately snap frozen in liquid nitrogen and stored at -80°C until processing for DNA isolation. Analysis was performed as described.⁽²⁸⁾ (See Supporting Methods for details.)

FLOW CYTOMETRY ANALYSIS

After blocking with an anti-Fc receptor antibody (cluster of differentiation [CD]16/32; BD Pharmingen, San Diego, CA) for 20 minutes at 4°C , the cells were incubated with specific fluorescence-labeled monoclonal antibodies at 4°C for 30 minutes. The following monoclonal antibodies were used: anti-CD11b (allophycocyanin [APC]-cyanine 7 dye [Cy7]), anti-CD11c (phycoerythrin [PE]-Cy7), anti-lymphocyte antigen 6 complex locus G6D (Ly6G) (Brilliant Violet 510 [BV510]), anti-Ly6C (APC), anti-F4/80 (fluorescein isothiocyanate [FITC]), anti-siglec H (PE), anti-CD1d tetramer (PE), anti-CD3 (PE-Cy7), anti-T-cell receptor beta chain (TCR β) (PE-Cy7), anti-CD4 (BV421), anti-CD8 (APC-Cy7), anti-natural killer (NK)1.1 (PE-Cy7), and anti-CD45 (BV510) (eBioscience, BD Pharmingen). The stained cells were analyzed using a fluorescence-activated cell-sorting Canto II (Becton Dickson, Rutherford, NJ), and the data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was extracted and purified from liver tissue using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands), and complementary DNA (cDNA) was synthesized with the purified RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Gene expression analysis was performed using the SYBR Green method or the TaqMan polymerase chain reaction (PCR) assay (Thermo Fisher Scientific) and a TaKaRa PCR Thermal Cycler Dice instrument (TaKaRa Bio, Shiga, Japan). All primers used for real-time PCR were purchased from FASMAC

(Kanagawa, Japan). The primer sequences are listed in Supporting Table S1.

ANALYSIS OF MYELOPEROXIDASE ACTIVITY AND ATP ASSAY

Liver tissues were homogenized to obtain 10% homogenate with normal saline, and myeloperoxidase (MPO) activity was measured with neutrophil MPO assay kits (Cayman Chemical; Ann Arbor, MI) according to the manufacturer's instructions. Livers were harvested and immediately frozen in liquid nitrogen; intrahepatic ATP was then measured using an ATP assay kit (ab83355; Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions.

PORTAL VEIN PLASMA SAMPLING FOR SCFA QUANTIFICATION

SCFA quantification of plasma was performed by liquid chromatography-tandem mass spectrometry (LC/MS-MS) at Shimadzu Excellence in Science (Kyoto, Japan).

GUT STERILIZATION AND FECAL MATERIAL TRANSPLANTATION

Mice were treated with broad-spectrum antibiotics through a nasogastric tube (500 μL /each) 3 times a week for 2 weeks. The following concentrations of antibiotics were used: ampicillin (6.7 g/L), neomycin (6.7 g/L), vancomycin (3.3 g/L), and metronidazole (6.7 g/L), as reported.⁽²⁹⁾ Our preliminary assessment (data not shown) and a previous study⁽³⁰⁾ revealed that large populations of bacteria were no longer detectable and that the total amount of bacterial genomic DNA was greatly reduced after treatment. In fecal material transplantation experiments, some mice were administered fecal material, derived from the Inu- or Cont-diet-fed mice, through a nasogastric tube (500 μL /time) 3 times a week for 1 week before ConA administration.

IN VIVO ASSESSMENT OF INTESTINAL PERMEABILITY

Mice were orally provided with 4,000 Da FITC-dextran (Sigma-Aldrich) through a nasogastric tube after fasting for 6 hours. Blood samples were collected from the tail vein at 4 hours. The concentration of

FITC from the obtained serum was measured using spectrophotometry according to the manufacturer's instructions. Serum from mice not administered with FITC-dextran was used to determine the background.

PRIMARY HEPATOCYTE ISOLATION AND MANIPULATION

WT mouse primary hepatocytes were prepared and isolated as described.⁽³¹⁾ Acetate and butyrate (from Sigma-Aldrich) prepared at 1 mM were used for incubation with primary hepatocytes for 16 hours before analysis as reported.⁽³²⁾

PPAR γ INACTIVATION THROUGH SMALL INTERFERING RNA

Mice fed a Cont- or Inu-diet were intravenously injected through the tail vein with control or Pparg small interfering RNA (siRNA; 1 mg/kg body weight) using InvivoFectamine 3.0 Reagent (Thermo Fisher Scientific) for liver-specific target gene knockdown at the start of specific diets. In *in vitro* experiments, control or Pparg siRNA was transfected into hepatocytes using Lipofectamine RNAiMAX (Thermo Fisher Scientific) as described.⁽³³⁾ Expression vectors were transfected into hepatocytes using Lipofectamine LTX Reagent according to the manufacturer's protocol.

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism 9.0.0 and expressed as the mean \pm SEM. The Mann-Whitney U test, unpaired Student *t* test, and analysis of variance were used as appropriate. Differences were considered statistically significant at $P < 0.05$.

Results

INU-DIET SIGNIFICANTLY DIMINISHES HEPATIC DAMAGE INDUCED BY ConA

First, we confirmed that the adjustment of the inulin-supplemented diet (Supporting Fig. S1A) did not induce any difference in body weight between mice fed the different diets before the induction of ConA hepatitis (Supporting Fig. S1B).

After ConA administration (Fig. 1A), diminished hepatic damage was observed both histologically (Fig. 1B) and serologically (Fig. 1C; Supporting Fig. S1C) in the Inu-diet group compared to the Cont-diet group. The levels of gene expression of IFN- γ and TNF- α , the main known effector cytokines in ConA-induced liver injury, along with inducible nitric oxide synthase and inflammatory chemokine monocyte chemoattractant protein 1 were significantly down-regulated in the livers of mice in the Inu-diet group (Fig. 1D). Intrahepatic neutrophil accumulation, as demonstrated by MPO activity assays (Fig. 1E) and flow cytometric analyses (CD11b⁺Ly6G⁺; Fig. 1F, with gating strategies shown in Supporting Fig. S2A), was also significantly diminished. No significant differences in frequencies of intrahepatic T, NK, and NKT cells were observed between the two groups (Supporting Fig. S2B), except for higher frequencies in CD11b⁺CD11c⁺ myeloid cells (Fig. 1F) and Helios⁺ thymus-derived natural regulatory T cells (Tregs) in the Inu-diet group (Supporting Fig. S2C). Suppressive effects of ConA-induced acute liver injury by inulin supplementation in *IL10* KO mice (Supporting Fig. S2D) and *RAG2* KO mice pretreated with rIFN- γ and rTNF (Supporting Fig. S2E) suggested a minimum contribution of IL-10 and T cells to the suppression. Collectively, the diminished intrahepatic accumulation of MPO-producing neutrophils after ConA administration may have principally contributed to this protective effect. The protective effect of inulin supplementation was confirmed in another immune-mediated liver injury mouse model induced by the administration of α -galactosylceramide (Supporting Fig. S3A). This protective effect was not confirmed in mice with "acute" liver injury induced by the chemical CCl₄, a model that immune-mediated hepatocyte damage plays a less important role in the acute phase (Supporting Fig. S3B).

PROTECTIVE EFFECT OF THE INU-DIET AGAINST ConA-INDUCED HEPATITIS IS DEPENDENT ON DECREASED HEPATIC ATP BY PURINERGIC P2 RECEPTOR

ATP release guides neutrophil chemotaxis by purinergic P2 receptors.⁽³⁴⁾ Therefore, we further focused

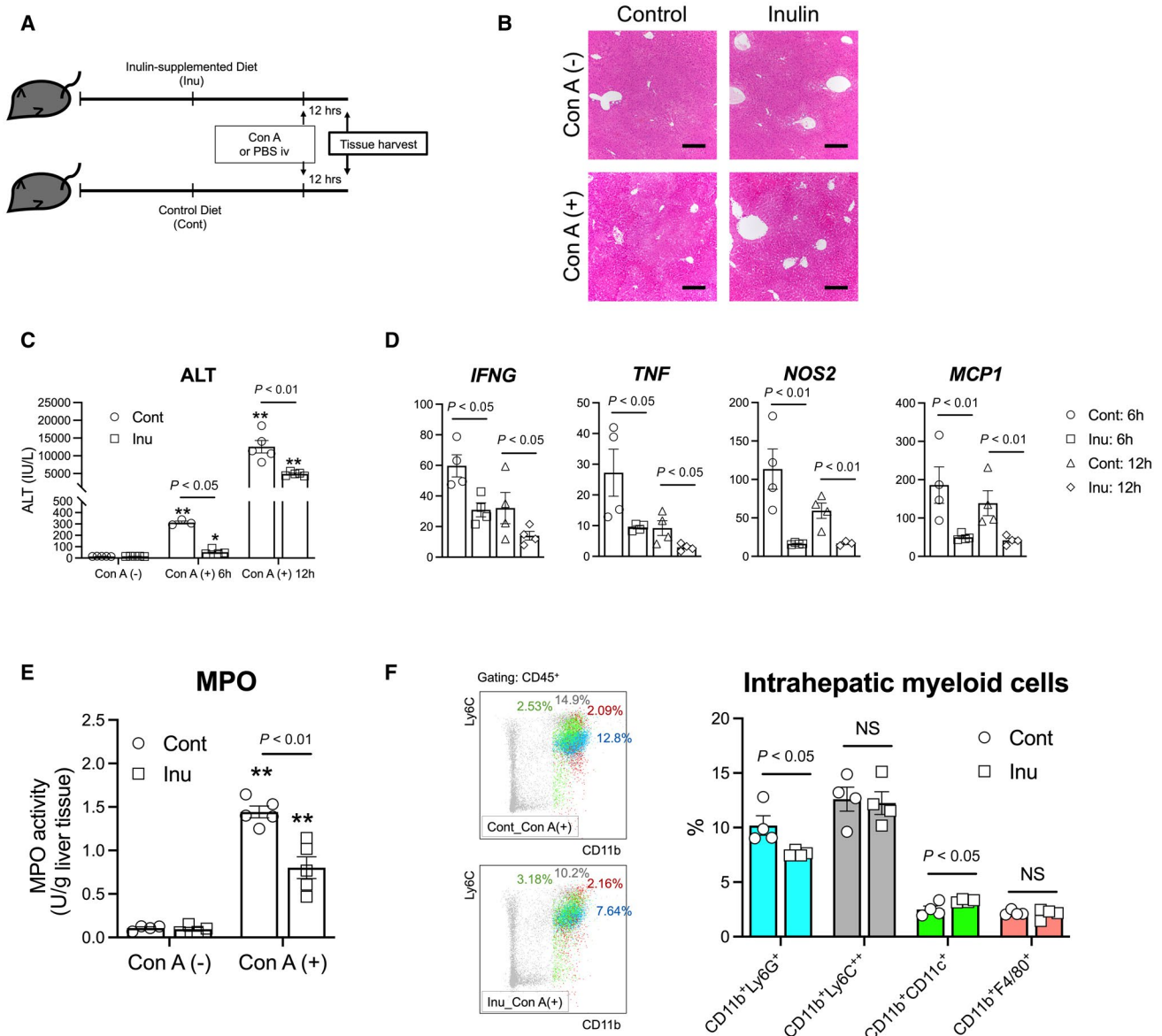


FIG. 1. Inulin-supplemented diet-fed mice show diminished hepatic damage and reduced inflammation in ConA-induced hepatitis. (A) Study design. Mice fed a control or inulin-supplemented diet for 2 weeks were intravenously injected with PBS or ConA (20 mg/kg). Tissues were harvested 12 hours following the injection. (B) Representative photomicrographs of H&E-stained sections of the liver (magnification $\times 200$). (C) Serum ALT levels at indicated time points after ConA administration. (D) Arbitrary ratios of mRNA expression relative to *GAPDH* normalized to those treated with vehicle (PBS) for the whole liver. (E) MPO activity, demonstrated in units per gram of liver tissue. (F, left panels) Representative CD11b and Ly6C staining on total isolated mononuclear liver cells. Colors representing each subgroup are as follows: red, CD11b⁺F4/80⁺; green, CD11b⁺CD11c⁺; blue, CD11b⁺Ly6G⁺; gray, CD11b⁺Ly6C⁺⁺. (F, right panel) Percentages of intrahepatic CD11b⁺ myeloid cells. Data are shown as mean \pm SEM. Statistical significance compared between ConA and PBS is shown as * $P < 0.05$ or ** $P < 0.01$, and those between Con- and Inu-diet groups are indicated. Abbreviations: h/hrs, hours; IFNG, interferon- γ ; iv, intravenous; MCP1, monocyte chemotactic protein 1; NOS2, nitric oxide synthase 2; PBS, phosphate-buffered saline.

on whether the diminished intrahepatic accumulation of MPO-producing neutrophils after ConA administration observed in the Inu-diet group (Fig. 1E,F) is associated with hepatic ATP levels. We confirmed

that preconditioning with Inu-diet for 2 weeks significantly decreased hepatic ATP accumulation in mice (Supporting Fig. S4); the effect lasted following ConA administration (Fig. 2A). Co-administration of suramin,

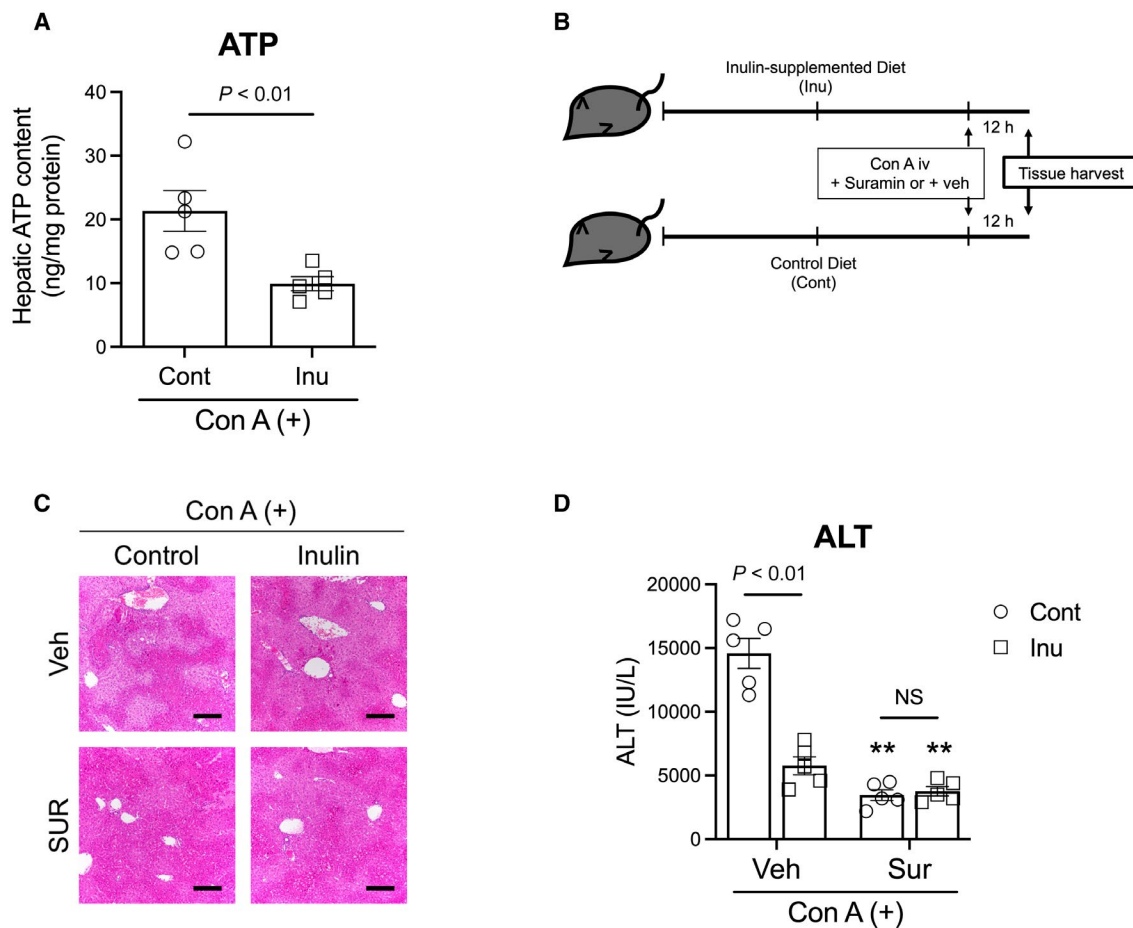


FIG. 2. The protective effect of an inulin-supplemented diet against ConA-induced acute hepatitis is dependent on the purinergic P2 receptor. (A) Hepatic ATP levels evaluated at 12 hours after ConA injection in mice fed a control or inulin-supplemented diet for 2 weeks in advance. (B) Study design. Mice fed a control or inulin-supplemented diet for 2 weeks were intravenously injected with ConA. Suramin (200 $\mu\text{g/g}$) or the vehicle were co-administered with ConA. Tissues were harvested 12 hours after ConA injection. (C) Representative photomicrographs of H&E-stained sections of the liver (magnification $\times 200$) and (D) serum ALT levels. Data are shown as mean \pm SEM. Statistical significance compared between suramin and vehicle is shown as $**P < 0.01$, and those compared between Con- and Inu-diet groups or otherwise specified are indicated. Abbreviations: h, hour; SUR, suramin; veh, vehicle.

a purinergic P2 receptor antagonist, inhibited the protective effect of the Inu-diet against ConA-induced immune-mediated liver injury (Fig. 2B-D). These results collectively suggest that decreased hepatic ATP accumulation plays a critical role in the protective effect of inulin supplementation against immune-mediated liver injury.

GUT MICROBIOTA COMPOSITION MODIFIED BY THE INU-DIET IS CRUCIAL FOR ALLEVIATING ConA-INDUCED HEPATITIS

Inulin is a well-known prebiotic that modifies gut microbiota composition. To understand how inulin

supplementation alters liver function, we profiled the gut microbiota by 16S ribosomal RNA (rRNA) sequencing. After 2 weeks of preconditioning with the inulin-supplemented diet, the 16S rRNA metagenomic analysis of fecal materials demonstrated differences in clustered relative abundance at the family level (Fig. 3A) and in the results of principal coordinate analysis of weighted UniFrac (Fig. 3B) compared to the Cont-diet. A minor but significant decrease in the diversity of the gut microbiota, demonstrated by comparing bacterial operational taxonomic units (OTUs), was also observed (Fig. 3C). With characterization of the microbiome by analyzing results of 16S rRNA high-throughput gene sequencing and linear discriminant

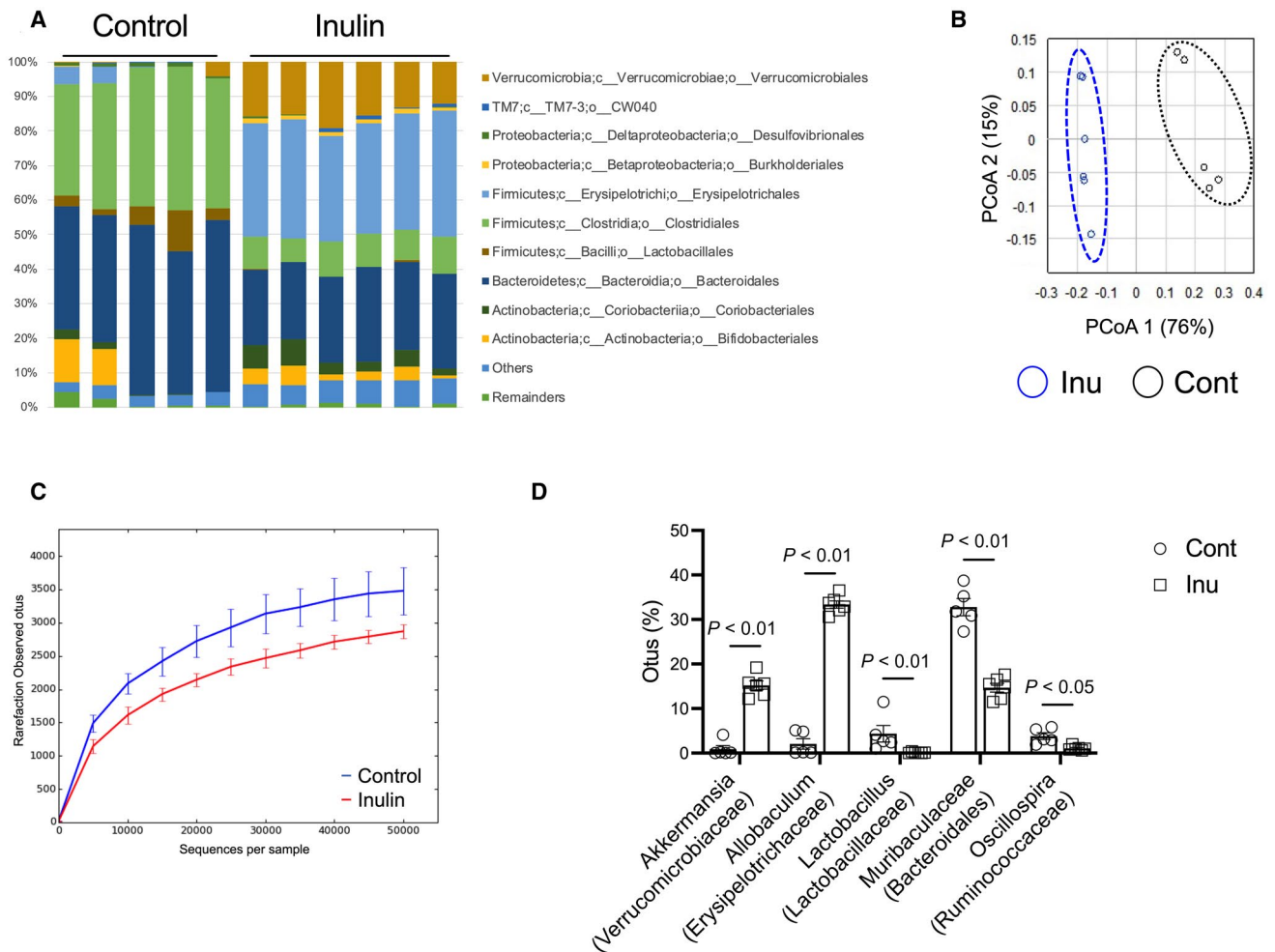


FIG. 3. Preconditioning with an inulin-supplemented diet results in significant compositional changes in the gut microbiota. (A) High-throughput sequencing of 16S rRNA of fecal bacterial DNA analysis demonstrated the composition and relative abundance of the gut microbiota at the order level in collected feces derived from Cont- and Inu-diet-fed mice after 2 weeks of diet preconditioning. (B) Principal coordinate analysis based on weighted UniFrac analysis of the bacterial community structures. (C) Rarefaction curve of observed OTUs. (D) Relative abundance of specific taxa analyzed in the microbiome of mice as collected in A. Data are shown as mean \pm SEM. Statistical significance is indicated. Abbreviations: PCoA, principal coordinate analysis; TM7, Saccharibacteria.

analysis effect size analysis, we noticed that the genus *Akkermansia* (Family *Verrucomicrobiaceae*) and genus *Allobaculum* (Family *Erysipelotrichaceae*) with a dominating presence increased significantly in the Inu-diet group compared to the Cont-diet group (Fig. 3D). We also noticed that gut sterilization with a combination of four unabsorbable antibiotics for 2 weeks before ConA administration, as illustrated in Fig. 4A, removed the protective effect of the inulin-supplemented diet against immune-mediated liver injury (Fig. 4B,C). However, fecal material transfer of

Inu-diet to gut-sterilized mice fed the Cont-diet, as illustrated in Fig. 4D, did not reproduce the protective effect of inulin supplementation against immune-mediated liver injury (Fig. 4E). In addition, intestinal permeability, assessed by *in vivo* FITC-dextran analyses, showed no significant differences between the Inu- and Cont-diet with or without ConA administration (Fig. 4F). These results suggest that gut microbiota is needed to achieve the protective effect by inulin supplementation while gut microbiota alone is insufficient.

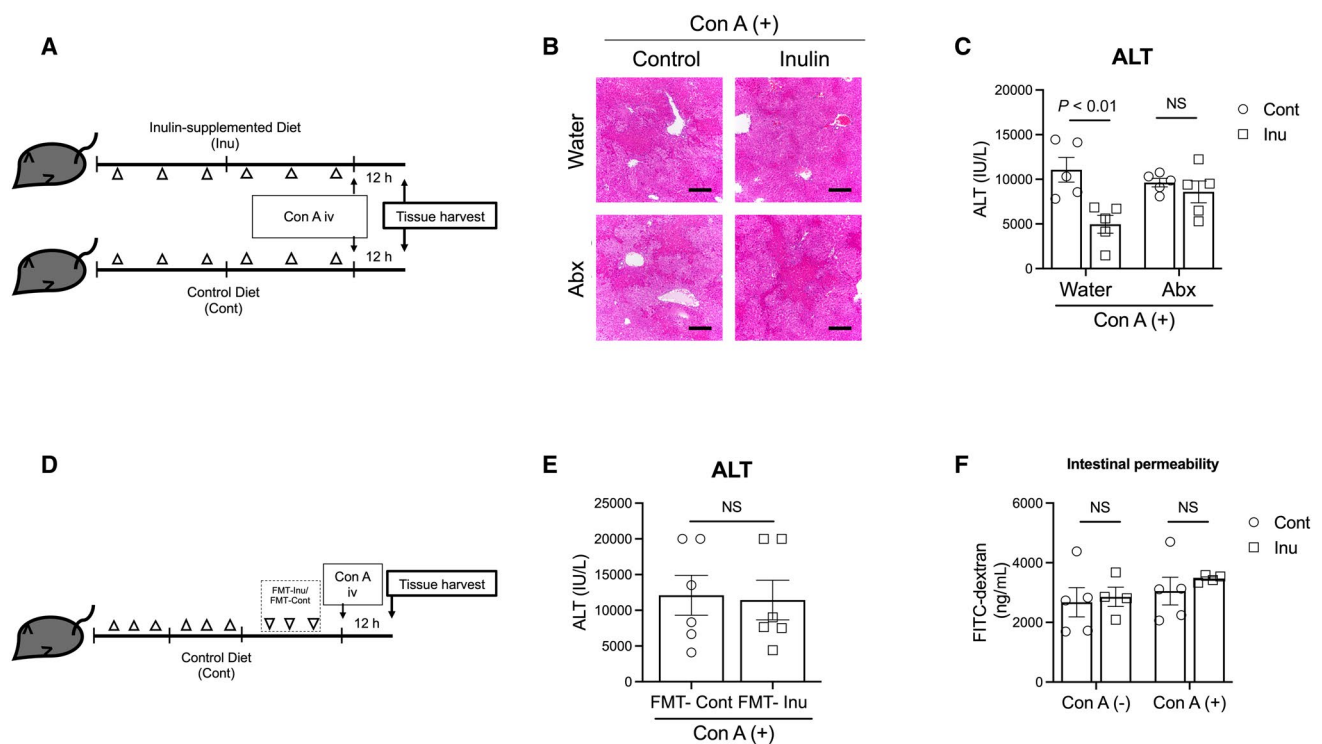


FIG. 4. Protective effect of an inulin-supplemented diet against ConA-induced acute hepatitis is dependent on the gut microbiota. (A) Study design. For gut sterilization, control or inulin-supplemented diet-fed mice were treated with water or broad-spectrum antibiotics through a nasogastric tube (500 μ L/each) 3 times a week for 2 weeks (open triangle), followed by ConA injection. Tissues were harvested 12 hours after ConA injection. (B) Representative photomicrographs of H&E-stained sections of the liver (magnification $\times 200$). (C) Serum ALT levels 12 hours after ConA administration in experiments represented in A. (D) Study design. After gut sterilization for 2 weeks (open triangle), mice were administered with fecal material derived from the Inu- or Cont-diet-fed mice through a nasogastric tube (500 μ L/time) 3 times a week for 1 week (reversed open triangle), followed by ConA injection. Tissues were harvested 12 hours after ConA injection. (E) Serum ALT levels 12 hours after ConA administration in experiments represented in D. (F) FITC-dextran evaluated intestinal permeability in experiments represented in Fig. 1A. Data are shown as mean \pm SEM. Abbreviations: Abx, antibiotics; FMT, fecal microbiota transplantation; h, hours; iv, intravenous.

PORTAL PLASMA WITH COMPOSITIONAL CHANGES OF SCFAs MODIFIED BY THE INU-DIET ALLEVIATES ConA-INDUCED HEPATITIS

Inulin increases the concentration of SCFAs in the gut^(19,35); thus, we conducted a quantificational analysis using LC/MS-MS of compositions of SCFAs in the portal plasma. Significantly higher concentrations of acetate, isobutyrate, and butyrate were noticed in the portal plasma collected from mice in the Inu-diet group compared to the Cont-diet group (Fig. 5A). Interestingly, the systemic preconditioning of portal plasma from mice fed the inulin-supplemented diet significantly alleviated ConA-induced immune-mediated

liver injury in mice fed the Cont-diet (Fig. 5B-D). We next examined the potential roles of SCFA receptors in ConA-induced liver injury. However, the protective effect of the Inu-diet against ConA-induced immune-mediated liver injury was not abolished in *GPR41* KO and *GPR43* KO mice compared to WT mice (Fig. 5E).

SCFA-PPAR γ -UCP2 AXIS DECREASES ATP ACCUMULATION IN HEPATOCYTES

We further focused on possible GPR41/GPR43-independent protective roles of SCFAs against ConA hepatitis. SCFAs, such as butyrate and propionate, serve as potent agonists of PPAR γ . We confirmed

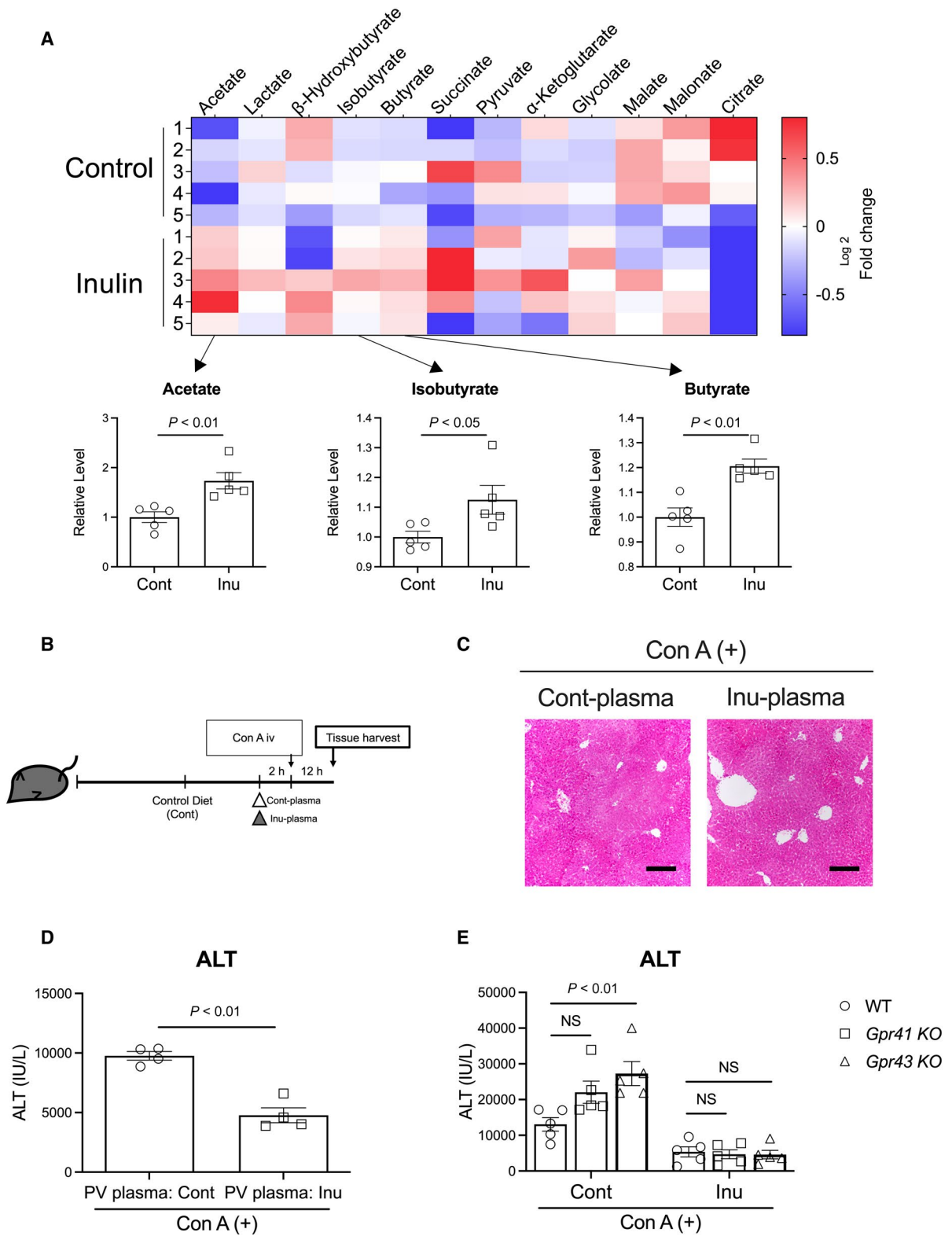


FIG. 5. Preconditioning with an inulin-supplemented diet results in significant compositional changes of portal plasma SCFAs. (A) Quantification of the SCFAs of plasma from the portal blood of mice fed a control diet or inulin-supplemented diet for 2 weeks analyzed by LC/MS-MS. SCFAs are presented with significant difference between groups specified in column graphs. (B) Study design. Control diet-fed mice were intravenously administered with plasma of portal vein blood (200 μ L/body) derived from control diet-fed or inulin-supplemented diet-fed mice 2 hours before ConA injection. Tissues were harvested 12 hours after ConA injection. (C) Representative photomicrographs of H&E-stained sections of the liver (magnification \times 200) and (D) serum ALT levels. (E) Serum ALT levels evaluated in experiments represented in Fig. 1A comparing WT, *GPR41* KO, and *GPR43* KO mice. Data are shown as mean \pm SEM. Statistical significance is indicated. Abbreviations: h, hours; iv, intravenous; PV, portal vein.

that preconditioning with an Inu-diet for 2 weeks significantly up-regulated hepatic *Pparg2*. Moreover, the expression of *Ucp2*, which uncouples mitochondrial ATP synthesis downstream of PPAR γ , also significantly increased (Fig. 6A). Fat-specific protein 27 (*Fsp27*), a hepatic steatosis mediator downstream of PPAR γ signaling,⁽³⁶⁾ was also up-regulated (Fig. 6A). These findings are implicative of global changes in hepatic energy utility in Inu-diet-fed mice. We also confirmed similar results in primary hepatocytes supplemented with acetate or butyrate *in vitro* (Fig. 6B). In addition, when the PPAR γ signal was abolished by siRNA (Fig. 6C), the intracellular ATP-reducing effects of acetate or butyrate were canceled in groups lacking PPAR γ *in vitro* (Fig. 6D). These results suggest that hepatic ATP content was diminished in an SCFA-PPAR γ -UCP2 axis-dependent manner.

Finally, by *in vivo* pretreatment with siRNA before ConA administration, we demonstrated that the protective effect of inulin supplementation against MPO-producing neutrophil infiltration and the subsequent immune-mediated liver injury was canceled as PPAR γ signaling was abolished (Fig. 7A-E). These results collectively suggest that inulin supplementation plays a critical role in the protective effect against immune-mediated liver injury by decreasing ATP accumulation through the SCFA-PPAR γ -UCP2 axis.

Discussion

In the current study, using the ConA-induced acute immune-mediated liver injury murine model, we demonstrated that water-soluble and fermentable dietary fiber inulin supplementation significantly alleviated immune-mediated hepatocyte damage by (i) increasing the portal plasma contents of SCFAs, such as butyrate and acetate; (ii) activating the SCFA-PPAR γ -UCP2 axis to reduce hepatic ATP release; and

(iii) decreasing purinergic P2 receptor-mediated MPO-producing neutrophil hepatic infiltration. In addition, characteristic compositional changes of the gut microbiota were suggested. Our findings are summarized in Fig. 8. A possible causal relationship between dietary fiber deficiency and a predisposition to the disruption of liver tolerance and suffering tissue damage is implicated. These results also highlight dietary fiber and the SCFA-PPAR γ -UCP2 axis as potential therapeutic targets in immune-mediated liver injury.

Extracellular nucleotides, mainly ATP, participate in purinergic signaling pathways, a ubiquitous system of cell-to-cell communication.⁽³⁷⁾ Their pathogenetic roles, along with P2 receptors and ectonucleotidases that metabolize them, have been emphasized in ConA-induced acute hepatitis in mice.⁽³⁸⁾ ATP appears to be an important determinant of cell survival in the liver, particularly because the purinergic P2 receptor antagonist suramin markedly reduces the apoptosis of hepatocytes during Fas-induced murine fulminant hepatitis.⁽³⁹⁾ This current study is the first to demonstrate the SCFA-PPAR γ -UCP2 axis to function as an ATP-preserving apparatus that could possibly be manipulated through diet or gut microbiota to limit immune-mediated hepatic damage.

PPAR γ , a small family of ligand-activated transcription factors belonging to the nuclear receptor superfamily, along with PPAR α provide important links between microbiota and host organs, including the gut, liver, skeletal muscle, and adipose tissue, to orchestrate energy metabolism (glucose and lipid), inflammation, cell proliferation, and circadian rhythm.⁽⁴⁰⁾ Iannucci et al.⁽³²⁾ demonstrated that the SCFA-PPAR γ -UCP2 axis is crucial for cell-autonomous autophagic flux in hepatocytes *in vitro* and that gut sterilization by chronic antibiotic treatment diminished basal hepatic autophagy in mice *in vivo*. In the current study, we provide novel evidence to extend knowledge at both ends, with the

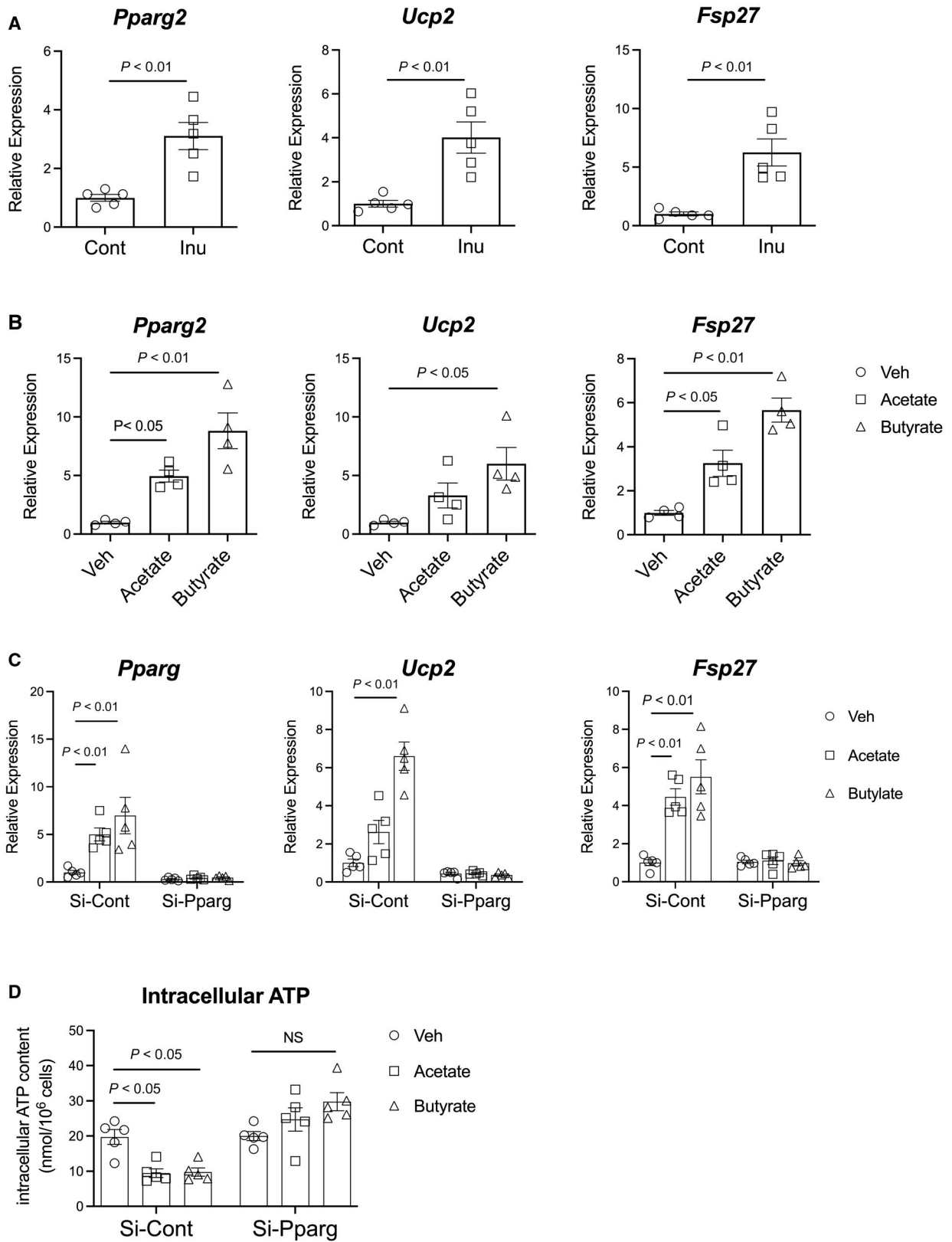


FIG. 6. SCFAs control ATP accumulation in hepatocytes through PPAR γ signaling. (A) Arbitrary ratios of mRNA expression relative to *GAPDH* for the whole liver of mice fed a control diet or inulin-supplemented diet for 2 weeks. (B) Arbitrary ratios of mRNA expression relative to *GAPDH* for the primary hepatocytes incubated with vehicle, 1 mM acetate, or 1 mM butyrate for 16 hours. (C) Arbitrary ratios of mRNA expression relative to *GAPDH* for the primary hepatocytes transfected with *Pparg* or control siRNA and incubated with vehicle, 1 mM acetate, or 1 mM butyrate for 16 hours. (D) Intracellular ATP levels (in nmol per 10⁶ cells) evaluated in primary hepatocytes transfected with *Pparg* or control siRNA and incubated with vehicle, 1 mM acetate, or 1 mM butyrate for 16 hours. Data are shown as mean \pm SEM. Statistical significance is indicated. Abbreviations: Si, siRNA; veh, vehicle.

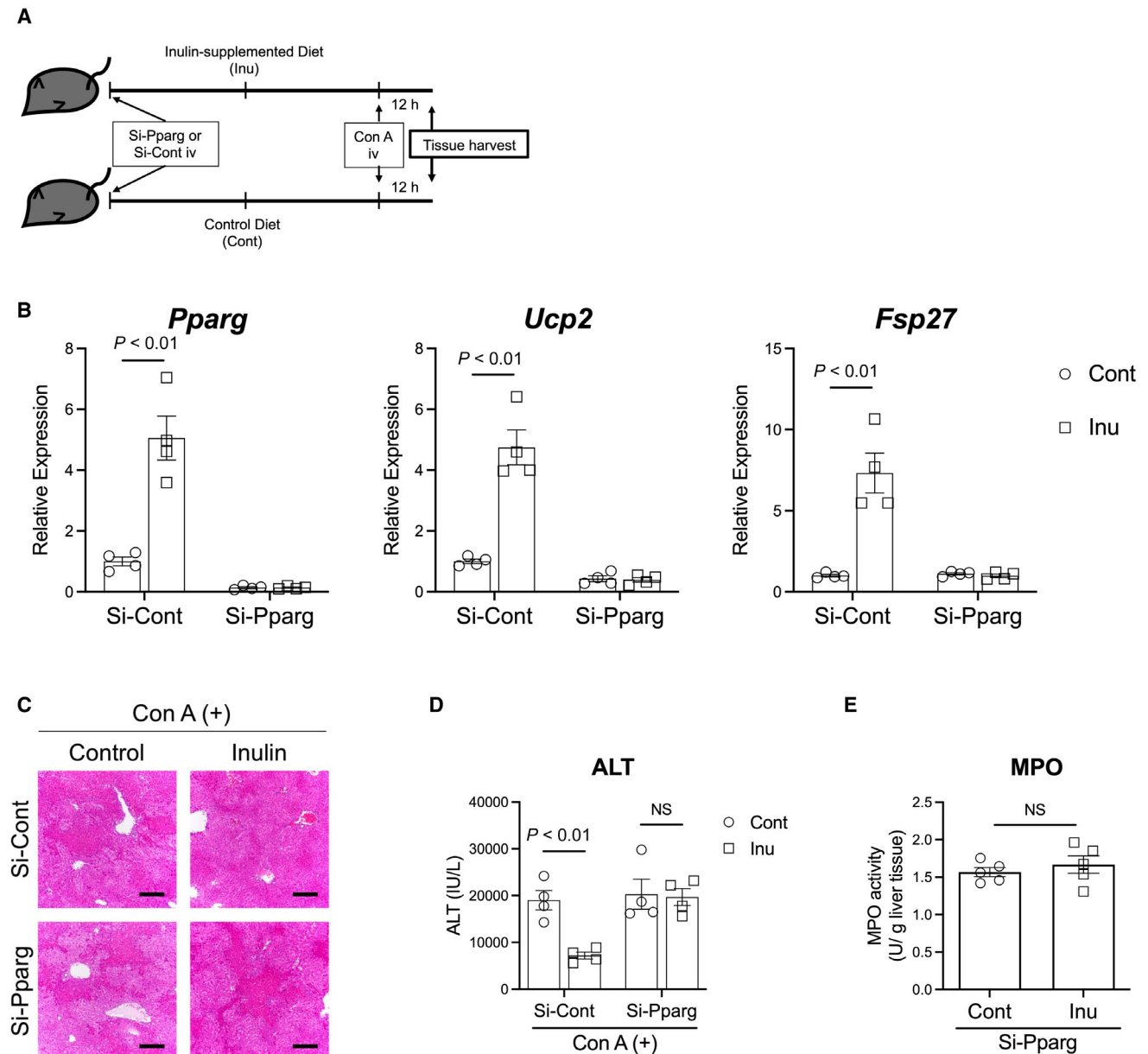


FIG. 7. The protective effect of an inulin-supplemented diet against ConA-induced acute hepatitis is dependent on PPAR γ signaling. (A) Study design. Mice intravenously administered with control or *Pparg* siRNA (1 mg/kg body weight) were fed control or inulin-supplemented diet for 2 weeks, followed by ConA injection. Tissues were harvested 12 hours after ConA injection. (B) Arbitrary ratios of mRNA expression relative to *GAPDH* for the whole liver of the indicated mice. (C) Representative photomicrographs of H&E-stained sections of the liver (magnification $\times 200$) and (D) serum ALT levels of the indicated mice. (E) MPO activity, demonstrated in units per gram liver tissue. Data are shown as mean \pm SEM. Statistical significance is indicated. Abbreviations: h, hours; iv, intravenous; Si, siRNA.

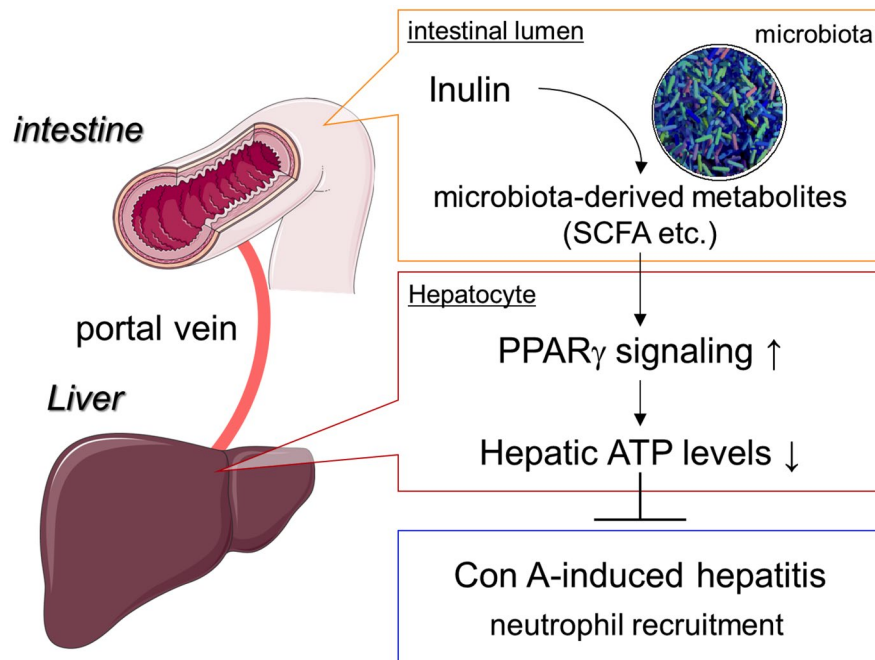


FIG. 8. Conceptual framework summarizing protective roles of an inulin-supplemented diet against ConA-induced immune-mediated acute hepatitis.

extracellular ATP–purinergic P2 receptor axis at the effector side and specific gut metagenomic characterization at the facilitator side to limit immune-mediated hepatic damage.

We noticed significant increases in observed OTUs in the genera *Akkermansia* and *Allobaculum* and a decrease in the genus *Lactobacillus* in mice fed an inulin-supplemented diet (Fig. 2D). *Akkermansia muciniphila*, the only isolated representative of the Verrucomicrobia phylum, protects against high-fat, diet-induced, metabolic disorders⁽⁴¹⁾ and ConA-induced acute hepatitis.⁽⁴²⁾ This bacterium is capable of producing acetate and propionate as a result of mucin degradation, which may in turn alter the relative abundance of SCFA-dependent microbes and regulate commensal reactions.⁽⁴³⁾ Although evidence for direct use of inulin by *A. muciniphila* is incomplete, oral supplementation of inulin increased its abundance in feces in patients with obesity and diabetes,⁽⁴⁴⁾ which is congruent to our observation. *Allobaculum* spp. (Erysipelotrichaceae phylum) are also SCFA producers and increase in prebiotic-treated, high-fat, diet-fed mice.⁽⁴⁵⁾ More surprisingly, decreased *Allobaculum* and increased *Lactobacillus* levels in the gut microbiota, in contrast to changes in inulin-supplemented

diet-fed mice, have been reported in a “humanized” mouse model of AIH through the immunization of human leukocyte antigen (HLA)-DR3 transgenic nonobese diabetic mice with cytochrome P450 2D6 (CYP2D6).⁽¹²⁾ These observations highlight a possible etiopathophysiological link between the disruption of liver immunologic tolerance (or predisposition to developing AIH) and environmental factors, such as diet and gut microbiota.

Although a study with human subjects showed improved intestinal permeability in healthy volunteers fed an inulin-enriched diet,⁽⁴⁶⁾ significant differences in intestinal permeability were not observed in the murine model used in this study (Fig. 3F). We also did not observe worsening of ConA-induced hepatitis in inulin-supplemented diet-fed mice deficient in *GPR41* and *GPR43* (Fig. 4E), which suggests sparse causal effects through these receptors. Furusawa et al.⁽⁴⁷⁾ reported that commensal microbe-derived butyrate induces the differentiation of peripherally generated colonic Tregs in high-fiber diet-fed mice; however, we did not notice a significant change in peripherally generated Tregs in the livers of Inu-diet-fed mice and instead a significant increase in Helios⁺ thymus-derived natural Tregs (Supporting Fig. S2C).

The protective effect against ConA-induced hepatitis was maintained in inulin-supplemented diet-fed *IL10* KO mice (Supporting Fig. S2D); Tregs might play only minor roles in the model used in the current study.

Inadequate consumption of inulin-like fructans may aggravate hepatic steatosis in patients with non-alcoholic fatty liver disease.⁽⁴⁸⁾ We also observed up-regulation of *Fsp27* (Fig. 6A,B), a direct mediator of PPAR γ -dependent hepatic steatosis,⁽³⁶⁾ in inulin-supplemented diet-fed mice, which may implicate a link between inulin and this clinical observation. Nonetheless, murine cholestatic liver cancers are also induced by dysregulated microbial fermentation of soluble fiber in toll-like receptor 5-deficient mice.⁽⁴⁹⁾ Caution is needed for the rational use of fermentable dietary fibers.

Neutrophils play important roles in ConA-induced immune-mediated liver injury, as demonstrated in this current study and a published report⁽³⁸⁾; however, their roles are ill-defined in human classical chronic AIH. How our findings could translationally link to human AIH, especially a severe acute-onset variant in which “centrilobular necrosis” is a pathognomonic feature,⁽⁵⁰⁾ should be investigated in the future.

In summary, these results highlight a previously unknown protective role of dietary inulin supplementation against immune-mediated acute liver injury in mice through the SCFA-PPAR γ -UCP2 axis. Further studies to validate and explore these findings may provide important insights for the ethiopathophysiological basis of AIH and possible therapeutic targets in the future.

Acknowledgment: We thank I. Kimura (Kyoto University, Kyoto, Japan) for providing *GPR41* and *GPR43* KO mice. We also thank M. Sadakata (EN Otsuka Pharmaceutical Co., Ltd., Iwate, Japan) for assisting with the metagenomic analysis. We thank Editage (www.editage.jp) for English language editing.

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