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Research Article

Ginsenoside F2 Restrains Hepatic Steatosis and Inflammation by Altering the Binding Affinity of Liver X Receptor Coregulators

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ABSTRACT

Background: Ginsenoside F2 (GF2), the protopanaxadiol-type constituent in *Panax ginseng*, has been reported to attenuate metabolic dysfunction-associated steatotic liver disease (MASLD). However, the mechanism of action is not fully understood. Here, this study investigates the molecular mechanism by which GF2 regulates MASLD progression through liver X receptor (LXR).

Methods: To demonstrate the effect of GF2 on LXR activity, computational modeling of protein-ligand binding, Time-resolved fluorescence resonance energy transfer (TR-FRET) assay for LXR cofactor recruitment, and luciferase reporter assay were performed. LXR agonist T0901317 was used for LXR activation in hepatocytes and macrophages. MASLD was induced by high-fat diet (HFD) feeding with or without GF2 administration in WT and LXRα$^{-/-}$ mice.

Results: Computational modeling showed that GF2 had a high affinity with LXRα. LXRE-luciferase reporter assay with amino acid substitution at the predicted ligand binding site revealed that the S264 residue of LXRα was the crucial interaction site of GF2. TR-FRET assay demonstrated that GF2 suppressed LXRα activity by favoring the binding of corepressors to LXRα while inhibiting the accessibility of coactivators. *In vitro*, GF2 treatments reduced T0901317-induced fat accumulation and pro-inflammatory cytokine expression in hepatocytes and macrophages, respectively. Consistently, GF2 administration ameliorated hepatic steatohepatitis and improved glucose or insulin tolerance in WT but not in LXRα$^{-/-}$ mice.

Conclusion: GF2 alters the binding affinities of LXRα coregulators, thereby interrupting hepatic steatosis and inflammation in macrophages. Therefore, we propose that GF2 might be a potential therapeutic agent for the intervention in patients with MASLD.

1. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) refers to a wide range of liver diseases including hepatitis, fibrosis, and hepatocellular carcinoma, caused by excessive fat accumulation [1,2]. Owing to the abuse of dietary intake, the prevalence of MASLD has increased up to 32.4% which is considered a global pandemic [3]. However, despite the surge in the need for an efficacious treatment of MASLD, the absence of approved treatment suggests that it is necessary to understand the more detailed mechanisms underlying MASLD

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development to discover therapeutic targets.

Liver X receptors (LXRs) are ligand-inducible transcription factors that belong to the nuclear receptor family and regulate not only intracellular cholesterol and lipid homeostasis but also inflammation [4]. There are two isoforms identified such as LXRα and LXRβ, in which LXRα is primarily expressed in the liver while LXRβ is expressed ubiquitously [5]. Once ligands such as oxysterols, the oxygenated cholesterol derivatives, bind to LXRs, they form heterodimers with the retinoid X receptors (RXRs) and then move into the nucleus to bind to LXR response element (LXRE) for initiating transcription [6,7]. The transcriptional activity of LXR is regulated by interactions with coregulators after ligand binding-dependent conformational changes. In detail, upon ligand binding, the corepressor complex is dissociated from LXR, followed by the interaction of the coactivator with LXR, whereas in the absence of ligands, corepressors including nuclear receptor corepressor (NCoR) and silencing mediators for retinoid and thyroid receptors interact with LXR to restrict transcription of target genes [8].

LXRs have been considered to play important roles in the development of MASLD. Activation of LXRα is known to induce the transcription of lipogenic pathway-related genes including sterol regulatory element binding protein-1c (SREBP1c) and fatty acid synthase (FASN) [9]. This indicates that LXRα influences the development of steatosis, as the increased expression of LXRs, SREBP1c, and FASN can be seen in the liver of patients with MASLD [10]. Additionally, LXRα is a key transcription factor in the differentiation of monocytes into monocyte-derived macrophages (MDM) in the liver and their identity formation [11]. On the other hand, LXRβ activates the phagocytosis of apoptotic cells through ATP binding cassette subfamily A member 1 (ABCA1), a major function of macrophages [12,13]. In non-alcoholic steatohepatitis (NASH), the LXR-mediated transcription of macrophage identity genes is selectively impaired depending on the altered binding of NASH-specific enhancers, leading to the formation of inflammatory lipid- and scar-associated macrophage phenotype [14].

Ginsenosides are the major active pharmacological components of *Panax ginseng*, and their beneficial functions have been reported in high-fat diet (HFD), lipopolysaccharide (LPS), and ethanol-induced liver injury [15–17]. In a previous report, the minor ginsenosides, which have small molecular weights, showed improved therapeutic effects through higher absorption rates in the gastrointestinal tract than major ginsenosides [18]. Among them, ginsenoside F2 (GF2) has been known to play important roles in various diseases such as cancer therapeutics, anti-obesity, and anti-inflammation [19–22]. Interestingly, a previous study using a GF2-enhanced mixture revealed that oral administration of the GF2 mixture decreased lipid accumulation by stimulating antioxidant pathway and reducing SREBP expression in the livers of high-fat, high-carbohydrate diet-fed mice [23]. However, the exact underlying mechanisms of GF2-mixture have not been explored clearly.

Here, we aim to understand the molecular mechanism of single compound GF2 in the pathogenesis of MASLD. We suggest the beneficial functions of GF2 against MASLD by regulating the binding of LXR coregulator and selectively repressing the target genes through altered transcriptional activity. Moreover, we provide concrete evidence that GF2 restrains hepatic lipogenesis and inflammatory response of MDM through *in vivo* experiments. Accordingly, we posit that GF2 could be a potential therapeutic agent for the intervention of MASLD.

2. Materials and methods

2.1. Preparation of GF2

GF2 (>95% purity) was purified from yeast using the following process; Isocratic high-performance liquid chromatography with microporous adsorption resin (Diaion® HP-20, Supelco Inc., Bellefonte, PA, USA), Solvent gradient high-performance liquid chromatography (YMC Ltd., Kyoto, Japan) with YMC-DispoPackAT (Particle size: 50μm) and Recycling Preparative high-performance liquid chromatography with JAIGEL-ODS-AP column (10 mm, 20 mm i.d., Japan Analytical Industry Co., Ltd., Tokyo, Japan).

2.2. Ligand binding domain prediction assay

Computational protein-ligand docking of GF2 to LXRα (PDB ID: 3IPQ) or LXRβ (PDB ID: 1PQC) was carried out using Autodock Vina v1.2.0. (Scripps Research, San Diego, CA, USA) [24] and PyMOL molecular graphics system v2.0. (Schrödinger, LLC, NY, USA).

2.3. Site-directed mutagenesis analysis and reporter assay

The pEGFP-C1 hLXRα mutants (A261F, E267F, and S264F) and pEGFP-C2 hLXRβ mutants (A274F, E280F, and S278F) were generated using a QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA, USA) and verified by DNA sequencing. For transfection, 3 μg of plasmid were prepared in OptiMEM with 6μl of Lipofectamine 2000 transfection reagent (Life Technologies, Carlsbad, CA, USA) and were transfected to 293T cells. To measure effects on LXR activity, LXRE-luciferase reporter assay was assessed by using the Dual-Glo® Luciferase Assay System (Promega™, Madison, WI, USA), and Renilla luminescence was measured. The luciferase activity was further normalized to a co-transfected internal control reporter.

2.4. Coregulators binding assay

LXR coregulators (coactivator and corepressor) binding was measured using LanthaScreen™ time-resolved fluorescence resonance energy transfer (TR-FRET) LXR alpha or beta Coactivator Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For detail, the recruitment of fluorescent-labeled coactivator peptides and displacement of corepressor peptides were measured by an increase or a decrease in the TR-FRET signal between the Tb-anti-GST antibody and the fluorescein-conjugated on coregulator peptide. 520 nm/490 nm TR-FRET ratio was calculated from TR-FRET signal detected by iMark™ Microplate Absorbance Reader.

2.5. Animals

Male C57BL/6J wild type (WT) and LXRα deficient ($LXR\alpha^{-/-}$) mice aged 8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). For inducing MASLD, a 60% fat diet (HFD) was fed for 12 weeks. For *in vivo* experiment, 50 mg/kg GF2 dissolved in 40% ethanol (Merck, Darmstadt, Germany) was diluted in olive oil and administered to mice. Mice were maintained on a 12-hour light-dark cycle in a specific-pathogen-free facility according to the guidelines of the Care and Use of Laboratory Animals published by the National Institutes of Health. The protocol for the animal experiment was approved by the Institutional Animal Care and Use Committee of the Korea Advanced Institute of Science and Technology (KA2013-36).

2.6. Clinical chemistry measurements

Serum was collected from the supernatant after centrifuging whole blood at 1,000 g for 10 min. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and liver triglyceride were measured using VetTest® Chemistry Analyzer (IDEXX Laboratory Inc., Westbrook, ME, USA). For the glucose or insulin tolerance test, glucose (2 g/ kg; Sigma-Aldrich) or 1 U/ kg insulin (Humalog, Lilly, Indianapolis, IN, USA) were injected into mice intraperitoneally after fasting for 16 h. Blood glucose level was measured once every 30 min from 0 to 150 min after glucose injection and for 2 hours after insulin injection by glucometer (Allmedicus, Anyang, South Korea).

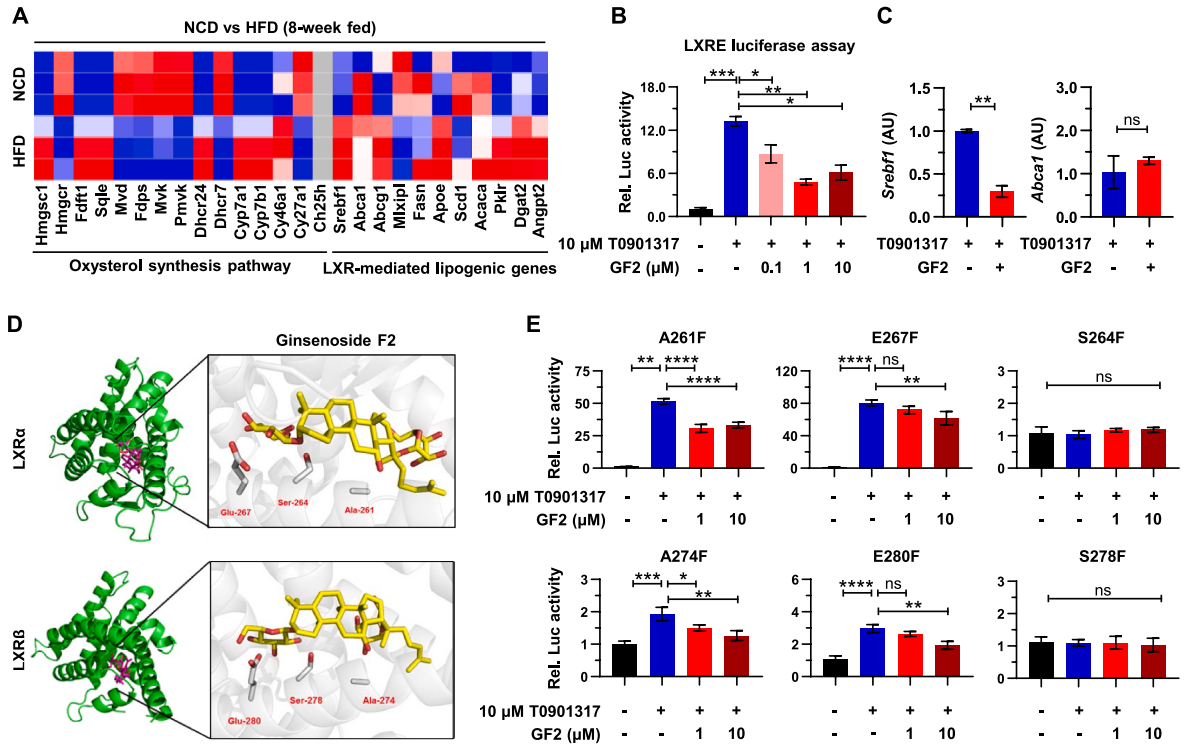

Fig. 1. GF2 reduces the LXR activity binding to the serine residue of the ligand binding pocket.
(A) Heatmap showing expression of genes related to oxysterol synthesis pathway and LXR-mediated lipogenesis in liver tissues of NCD- and HFD-fed mice. (B) Relative LXRE luciferase activity in 293T cells treated in the presence or absence of GF2 and 10 μM T0901317. (C) Relative mRNA expression of *Srebf1* and *Abca1* in 293T cells treated with 10 μM T0901317 in the presence or absence of 1 μM GF2. (D) Protein tertiary structures of LXRα and LXRβ with amino acid residues in the binding pocket as predicted sites of GF2 binding. (E) Relative LXRE luciferase activity in 10 μM T0901317- and/or GF2-treated 293T cells following amino acid substitution at the predicted binding site of LXRα and LXRβ. HFD, high-fat diet; NCD, normal chow diet; LXRE, LXR element; GF2, Ginsenoside F2. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns, not statistically significant.

2.7. Histological analysis

Mouse livers were fixed with 10% neutral buffered formalin or the OCT compound (Sakura, Tokyo, Japan), and embedded. Paraffin sections were stained with hematoxylin and eosin (H&E) for pathological diagnosis, while frozen sections were subjected to oil-red O staining (Sigma-Aldrich) to visualize lipid droplets.

2.8. Isolation of primary cells and cell culture

Mouse primary cells were isolated from perfused liver using Ethylene glycol tetra-acetic acid (EGTA) solution, and collagenase solution (0.075% of collagenase type I diluted in HBSS with 0.2% of DNase I). After perfusion, the liver was digested in a rotating incubator (37°C) for 20 min at 90 rpm with a digestion buffer (0.015% of collagenase type I in HBSS with 0.2% of DNase I) and filtered by a 70 μm cell strainer. After 50 g x 2 centrifugation, hepatocytes were purified by 50% percoll density gradient solution, while Kupffer cells were separated using Optiprep™ density gradient medium (Sigma-Aldrich) using the supernatant [25].

Bone marrow-derived macrophages (BMDMs) were differentiated from whole bone marrow cells isolated from the tibias and femurs of WT and LXRα$^{-/-}$ or LXRβ$^{-/-}$ mice by incubating for 5 days in RPMI medium containing 20 ng/ml M-CSF. To transdifferentiate into M1-type BMDM, 100 ng/ml LPS and 50 ng/ml IFN-γ were treated for 24 h.

Primary cells were primed with 200 μM of palmitate conjugated with 5% bovine serum albumin (BSA). For *in vitro* experiments, T0901317 and GF2 were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and treated to cells at 1 to 10 μM. Oil-red O-positive cells were quantified by Image J (NIH, Bethesda, Maryland, USA).

2.9. Comparative analysis of gene expression

RNA extracted from liver tissues or cells was reverse-transcribed to cDNA after which quantitative RT-qPCR was performed using TRIzol® reagent (Thermo Fisher Scientific), RNeasy Mini kit (Qiagen, Hilden, Germany), amfiRivert cDNA synthesis master mix (GenDEPOT, Houston, TX, USA), and SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). To quantify the transcription level, the mRNA expression levels of the target genes were normalized to *18s rRNA*. All samples were run in duplicate, and the relative gene expression was calculated as $2^{-\Delta CT}$, shown as a fold increase compared to control samples. The primer information for RT-qPCR is shown in Supplementary Table 1.

For bulk-RNA sequencing, extracted RNA was evaluated by Bioanalyzer2100 (Agilent, Santa Clara, CA, USA). Sequencing libraries were constructed by QuantSeq30′RNA-Seq Library Prep Kit FWD following the manufacturer's instruction and sequenced usSeq 500 platform (Illumina, San Diego, CA, USA). For visualization, heatmap was plotted as $\log_2$Fc.

2.10. Western blot analysis

Liver tissue protein samples were isolated using RIPA lysis buffer (30 mM pH 7.5 Tris, 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, 10% SDS, 10% glycerol), containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Purified proteins were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto the nitrocellulose membrane. After blocking with 5% skim milk, primary antibodies, SREBP-1 (MA5-16124, Thermo scientific), FASN (#3189, Cell signaling, Danvers, MA, USA), were added and incubated overnight at 4°C (1:1000), after which secondary antibodies were incubated for 1 h at 25°C (1:2000). Immunoreactive bands were visualized by

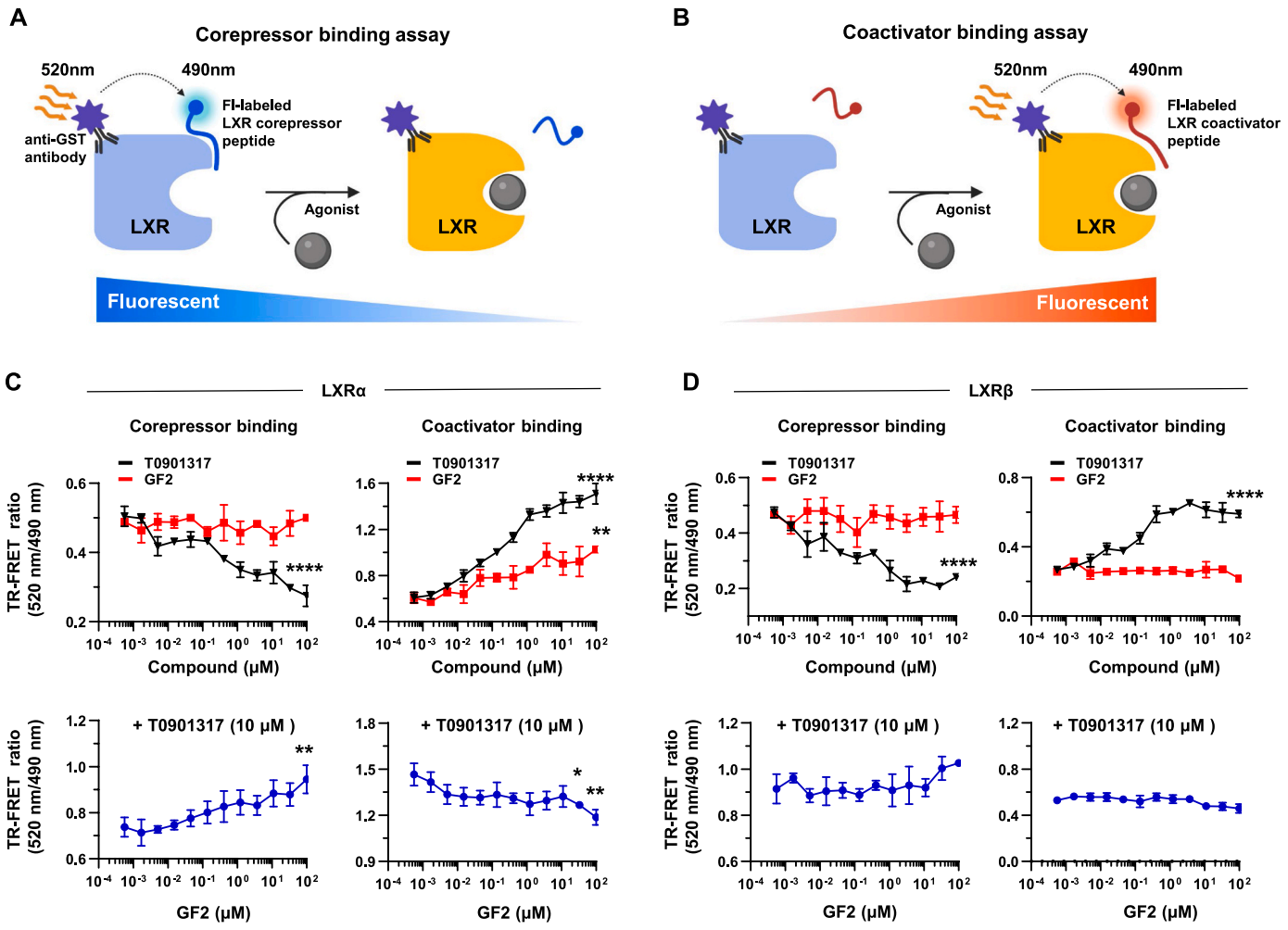

Fig. 2. GF2 maintains the binding affinity of LXRα corepressor

(A, B) Schematic diagram of a measurement strategy for corepressor displacement (A) and coactivator recruitment (B) from LXR by applying TR-FRET assay. (C, D) TR-FRET ratio (520 nm/490 nm) of corepressor and coactivator binding to LXRα (C) or LXRβ (D) when treated with serially increasing doses of T0901017 or GF2 (upper panel) and with GF2 in the presence of 10 μM T0901317 (lower panel). Data are presented as mean ± SEM. The significance of all values was verified with respect to the value at the lowest concentration of drug treatment. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

SuperSignal™ West Pico PLUS Chemiluminescent substrate (#34577, Thermo Fisher Scientific) and ImageQuant™ LAS 4000 (GE Healthcare, Chicago, IL, USA). The comparative amount of proteins was normalized with β-actin.

2.11. Flow cytometry analysis

Mouse liver mononuclear cells (MNCs) and Kupffer cells were labeled with fluorescence-tagged antibodies; eFluor 450-CD45 (clone 30-F11), FITC-F4/80 (clone BM8), APC-conjugated Ly-6G (clone 1A8), PE-CF594-conjugated SiglecF (clone E50-2440), APC-Cy7-conjugated CD11b (clone M1/70), PE-conjugated CD146 (clone ME-9F1). Fortessa™ cell analyzer and FlowJo software v10.8.1 (BD bioscience, San Jose, CA, USA) were used to identify each cell based on following markers: macrophages ($F4/80^{+}CD11b^{+}$), Kupffer cells ($F4/80^{hi}CD11b^{+}$), Eosinophils ($CD11b^{+}SiglecF^{+}$), neutrophils ($CD11b^{+}Ly6G^{+}$) in CD45+ cells, Live cells were determined with LIVE/DEAD™ fixable aqua dead cell stain kit (Thermo Fisher Scientific).

2.12. Statistical analysis

For statistical analyses, Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA) was used to perform data and shown as mean ± SEM. The statistical significance between the two groups or more than two groups was evaluated by unpaired Student t-test or one-way Analysis of Variance (ANOVA) with Tukey's or Dunnett's test for multiple comparisons, respectively. For RNA sequencing data, an adjusted p-value was used. A p-value <0.05 was considered to be statistically significant.

3. Results

3.1. GF2 reduces the transcriptional activity of LXR by binding to serine residue of the ligand-binding pocket in LXR

To explore the role of GF2 on LXR regulation, we first analyzed the alteration of hepatic LXR-related pathways by bulk-RNA sequencing of liver tissues of NCD- or HFD-fed mice for 8 weeks. In MASLD development, the expression of oxysterol synthetic pathway-related and LXR-mediated lipogenic genes was mostly up-regulated in the liver, suggesting that oxysterol-mediated LXR activation might play a role in the induction of hepatic steatosis and inflammation in the liver (Fig. 1A). Interestingly, diverse ginsenosides have similar chemical structures compared to oxysterols (Fig. S1A). Protopanaxadiol (PPD) and protopanaxatriol (PPT) ginsenosides consist of hydroxyl groups at either the carbon-3 and -20 positions or the −6 and −20 positions, respectively, on a steroid-like carbon backbone [26], suggesting the possible effect of

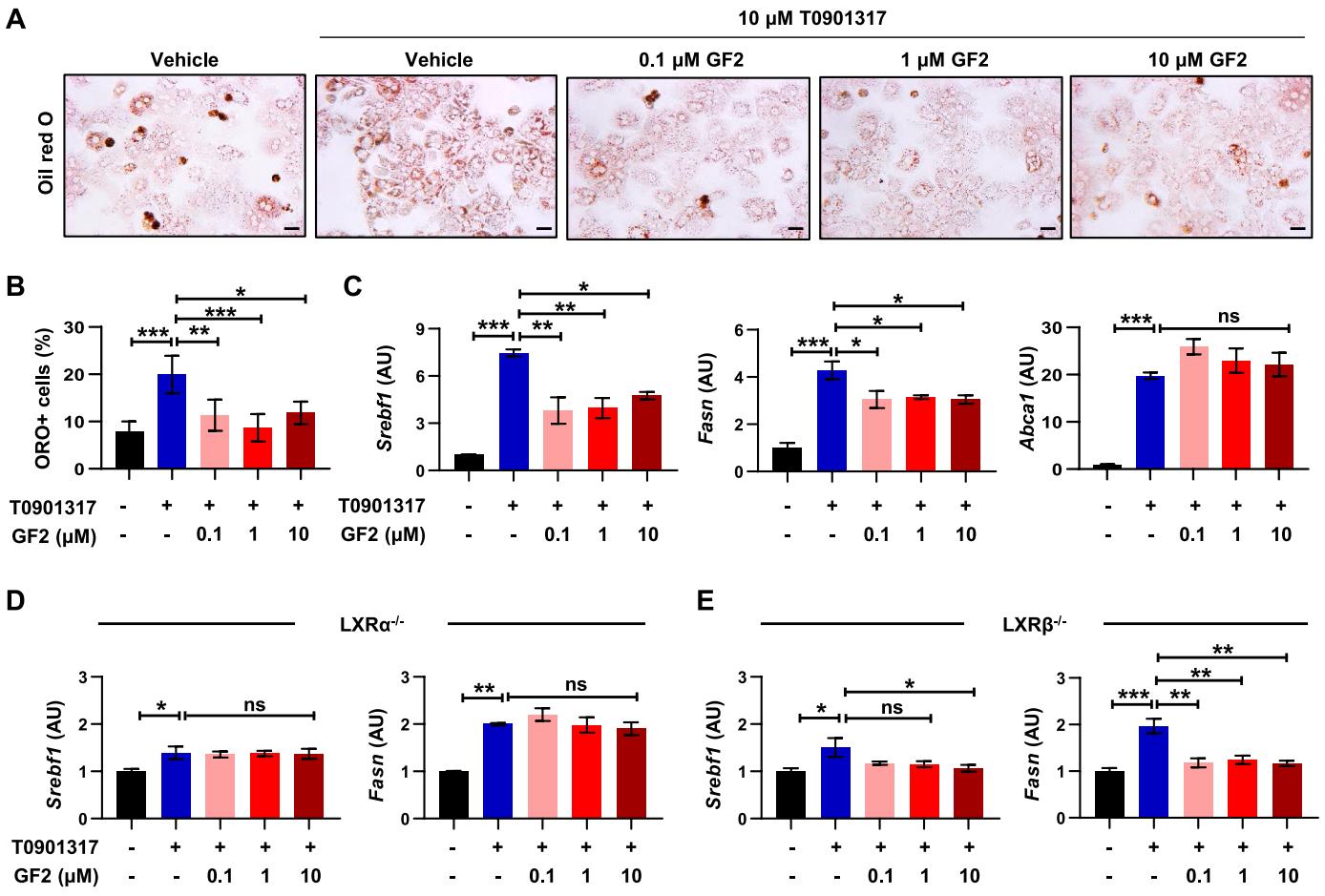

Fig. 3. LXRα-mediated lipogenesis is attenuated by GF2 in primary hepatocytes
(A, B) Representative oil red O staining and quantification of primary hepatocytes after GF2 treatment with T0901317. Scale bar = 20 μm. (C) Relative mRNA expression level of *Srebf1, Fasn, and Abca1,* after GF2 treatment with 10 μM T0901317 on primary hepatocytes. (D, E) Relative mRNA expression level of *Srebf1* and *Fasn* after GF2 treatment with 10 μM T0901317 on LXRα$^{-/-}$ (D) or LXRβ$^{-/-}$ (E) hepatocytes. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not statistically significant.

GF2 on LXR-mediated signaling pathways by replacing LXR ligands. To explore our hypothesis, we used LXRE-luciferase reporter assay to investigate whether treatment of minor ginsenosides with LXR agonist T0901317 affected LXR activity (Fig. S1B). Among ginsenosides, only GF2 treatment significantly reduced the luciferase activity and the expression of *Srebf1* but not *Abca1* (Fig. 1B and C, Fig. S1B). Next, the computational modeling of the receptor-ligand binding revealed that GF2 was predicted to interact with the residues at the ligand-binding pockets of both LXRα (Glu-267, Ser-264, and Ala-261) and LXRβ (Glu-280, Ser-278, and Ala-274) (Fig. 1D). After mutating the predicted ligand-binding sites of LXRα and LXRβ, the transfection efficiency was measured by GFP intensity and mRNA level of LXRs (Fig. S1C). Both mutations at the residues of LXRα (Ser-264) and LXRβ (Ser-278) lost luciferase activity as well as the inhibitory response of GF2, indicating that they are important ligand-binding sites of GF2 to LXRs (Fig. 1E).

3.2. GF2 alters the binding affinity of LXRα coregulators

To elucidate the more detailed inhibitory mechanism of GF2 after binding with LXRs, we investigated whether GF2 could regulate the transcription activity of LXR by regulating the binding of coregulators including coactivators or corepressors. To measure the binding affinities of coregulators to LXR, we adopted TR-FRET assay where fluorescein (Fl)-labeled corepressor or coactivator peptides could express fluorescence depending on the activation status of LXR through the conformational changes (Fig. 2A and B). As a result of the emission ratio in TR-FRET assay, LXR agonist T0901317 dissociated corepressor binding and sharply increased coactivator binding to both LXRs in a dose-dependent manner, whereas GF2 treatment did not change the corepressor binding to both LXRs but slightly increased the coactivator binding to LXRα (Fig. 2C and D, upper panels). However, in the co-treatments of T0901317 with GF2, GF2 was maintaining the corepressor binding and prevented the recruitment of coactivator to LXRs (Fig. 2C and D, lower panels). In addition, although GF2 could maintain the binding status of coregulators to both LXRs in the presence of T0901317, GF2 increased the binding of corepressor but dissociated the coactivator to LXRα in a dose-dependent manner, suggesting more specific activity to LXRα rather than LXRβ (Fig. 2C and D, lower panels). These results suggested that GF2 might inhibit the LXR activity by altering the binding affinities of LXR coregulators.

3.3. LXRα-mediated lipogenesis is reversed by GF2 in hepatocytes

Based on the regulatory mechanism of GF2 against LXRα, we investigated the inhibitory effect of GF2 on LXRα-mediated lipogenesis and fat accumulation in mouse primary hepatocytes. *In vitro* experiments, GF2 remarkably reduced fat accumulation induced by T0901317 to a similar degree to that of the control group (Fig. 3A and B). In addition, GF2 significantly decreased the mRNA expression of *Srebf1* and *Fasn* that were known as two representative target genes of LXR and master regulators of cellular lipogenesis (Fig. 3C). However, expression of *Abca1,* regulated by LXRβ activation, was unchanged by GF2

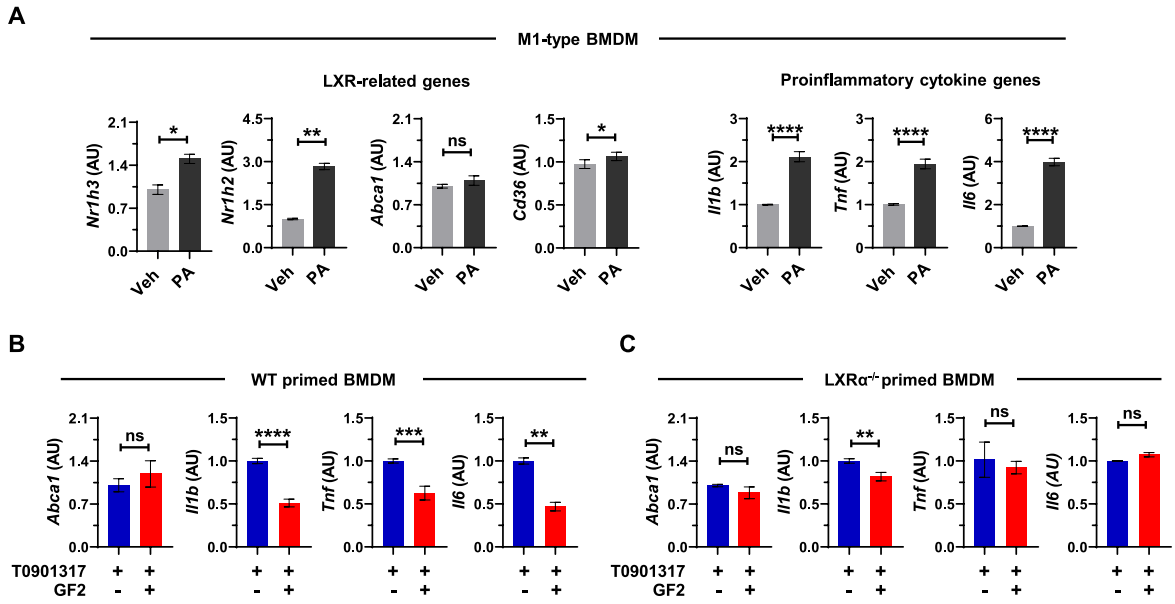

Fig. 4. Inflammatory response of macrophages is attenuated by GF2 in LXRα dependent manner.
(A) Relative mRNA expression level of LXRα (*Nr1h3*), LXRβ (*Nr1h2*), LXR target genes (*Abca1, Cd36*), and pro-inflammatory cytokines (*Il1b, Tnf,* and *Il6*) after PA treatment to M1-type BMDMs. (B, C) Relative mRNA expression level of *Abca1* and proinflammatory cytokines after GF2 treatment with T0901317 in primed BMDM from WT (B) and LXRα$^{-/-}$ (C) mice. PA, palmitic acid; BMDM, bone marrow-derived macrophage. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns, not statistically significant.

(Fig. 3C), indicating that GF2 may selectively regulate the transcriptional activity of LXRα. Next, to confirm the LXRα-dependent effects of GF2 in hepatic lipogenesis, T0901317 and GF2 were treated to primary hepatocytes isolated from LXRα- and LXRβ-deficient mice. qRT-PCR analyses revealed that GF2 treatment significantly suppressed mRNA expression of *Srebf1* and *Fasn* in LXRβ-deficient hepatocytes, but not in LXRα-deficient hepatocytes (Fig. 3D and E), suggesting that GF2 has an anti-lipogenic effect in hepatocytes through LXRα.

3.4. GF2 treatment suppresses the activation of pro-inflammatory macrophages through LXRα

As steatotic conditions are known to induce pro-inflammatory environments by recruiting and activating immune cells, especially macrophages [27,28], the possible effects of GF2 on macrophage activation were investigated *in vitro*. To mimic inflammatory macrophages in HFD-fed mouse liver, pro-inflammatory (M1-type) BMDMs were treated with palmitic acid (PA), referred to as primed BMDMs, and then were investigated for the expression of LXR-related genes and pro-inflammatory cytokine. qRT-PCR analysis showed that PA treatment increased the expression of genes such as *Nr1h3* (encoding LXRα), *Nr1h2* (encoding LXRβ), *Il1b, Tnf, Il6*, and *CD36,* but not in *Abca1*. (Fig. 4A). However, when primed BMDMs cultured with T0901317 ± GF2, GF2 treatment reduced the mRNA expression of pro-inflammatory cytokine genes (*Il1b*, *Tnf*, and *Il6*), but not *Abca1* (Fig. 4B). Notably, the anti-inflammatory effects of GF2 were not observed in the LXRα-deficient primed BMDMs (Fig. 4C). Consistently, GF2 treatment suppressed PA-induced activation of WT and LXRβ-deficient Kupffer cells whereas this was not observed in LXRα-deficient Kupffer cells (Fig. S2A-S2D). These results suggested that GF2 could reduce the expression of pro-inflammatory cytokines in both primed macrophages and Kupffer cells through LXRα, leading to the alleviation of hepatic inflammation.

3.5. GF2 alleviates MASLD and insulin resistance in mouse liver by downregulating hepatic lipogenesis and inflammation

To investigate the preventive effects of GF2 in the progression of MASLD *in vivo*, WT mice were fed HFD for 6 weeks and then orally administered 50mg/kg of GF2 once a day with HFD feeding for additional 6 weeks (Fig. S3A). As anticipated, the body and liver weight of mice were lower in the GF2-treated group than those of the vehicle (Veh)-treated group (Fig. S3B, S3C). Consistent with this, although ALT and AST levels were similar in both mice, liver gross, histological analyses, and measurements of hepatic triglyceride levels revealed that hepatic fat accumulation was significantly decreased in GF2-treated mice (Fig. 5A, 5B, S3D, S3E). In addition, glucose and insulin tolerance were ameliorated along with decreased weights of epididymal fat, sizes of adipocytes, and numbers of crown-like inflammatory foci (Fig. 5C, S3F). Moreover, the relative levels of FAS (*Fasn*) and cleaved-SREBP1 (*Srebf1*), as well as *Nr1h3* and *Nr1h2*, were also significantly decreased (Fig. 5D and E). Furthermore, F4/80^{+} hepatic macrophage infiltration was significantly reduced in the livers of GF2-fed mice compared to those of the controls (Fig. 5F). The number of immune cells infiltrating into the liver and the expression of genes encoding inflammatory cytokines (*Il1b*, *Tnf*, and *Il6*) were markedly decreased in the liver MNCs (Fig. 5G, S3G). Therefore, GF2 ameliorated not only HFD-induced hepatic *de novo* lipogenesis and insulin resistance but also inflammatory response in mice.

3.6. LXRα deficiency abolishes the effect of GF2 on steatosis and inflammation in MASLD

Next, we attempted to validate whether GF2 exhibits LXRα-mediated anti-steatosis and anti-inflammatory effects *in vivo*. When LXRα-deficient mice were fed HFD with GF2 administration, there were no changes in body and liver weights (Fig. 6A). Subsequently, with liver gross and histological analysis, accumulation of hepatic triglyceride and the expression of lipogenesis-related genes (*Srebf1* and *Fasn*) in liver tissue were similar in both groups (Fig. 6B–D). In addition, no significant changes in the numbers of infiltrating immune cells including macrophages as well as the expression of inflammatory genes were observed (Fig. 6E and F). Collectively, we suggest that GF2 executes anti-steatosis and anti-inflammatory functions in MASLD by enhancing the binding of corepressor to LXRα, thereby selectively regulating the transcription of target genes.

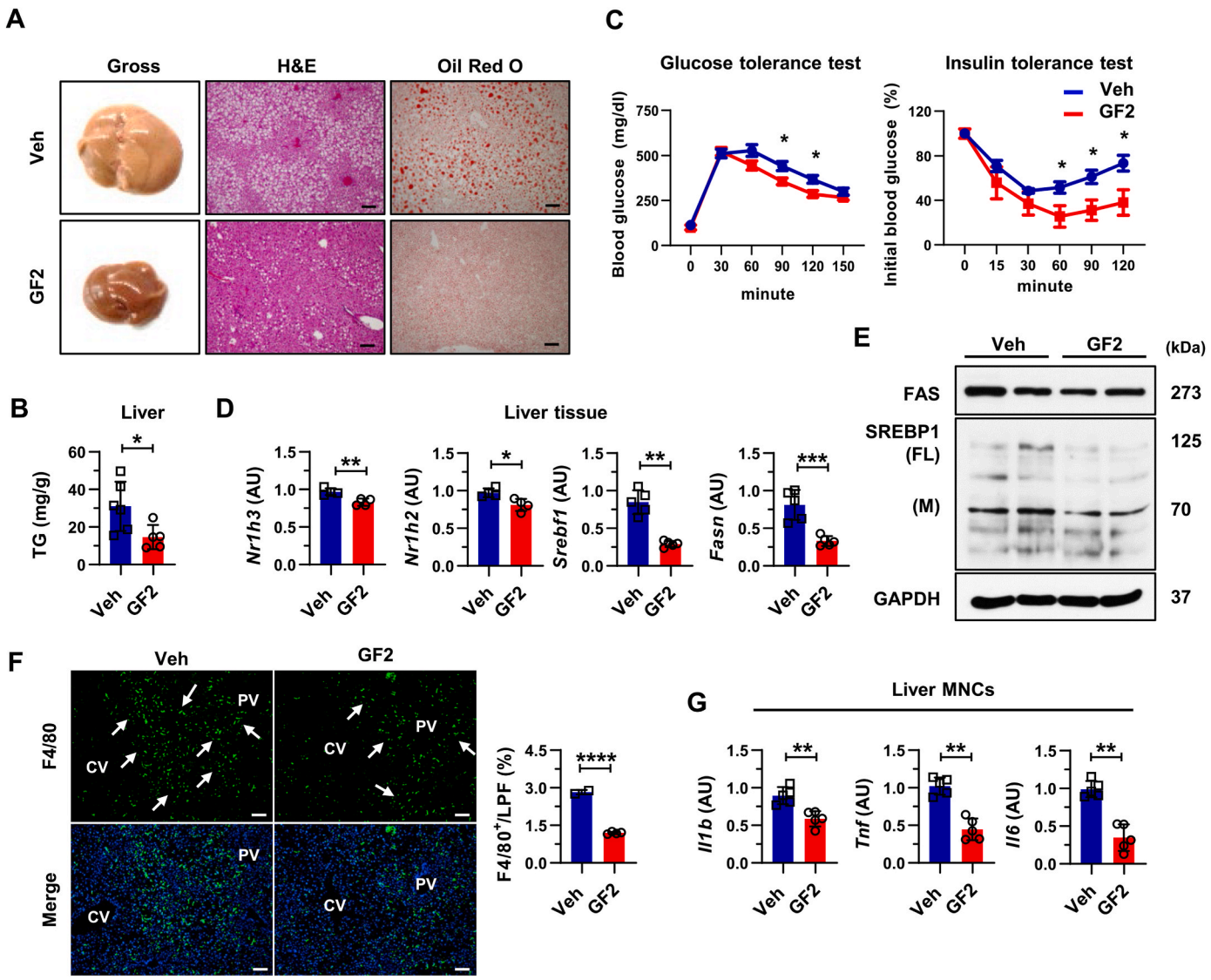

Fig. 5. GF2 alleviates MASLD by downregulating lipogenesis and inflammatory response.
Physiological and molecular analyses of livers from WT mice fed with HFD for 12 weeks with or without GF2 administration (n = 6/group). (A) Representative H&E and oil red O (ORO) staining in liver sections. Scale bar = 100 μm. (B) Hepatic triglyceride level, (C) blood glucose level in glucose tolerance test (GTT) and insulin tolerance test (ITT). (D) Relative mRNA expression level of *Nr1h3, N1rh2,* and lipogenesis-related LXR target genes (*Srebf1 and Fasn*). (E) Immunoblot of SREBP1 (FL, Full length; M, mature form) and FASN. (F) Immunofluorescence staining of hepatic macrophages with F4/80 in frozen liver tissue. Scale bar = 100 μm. (G) Relative mRNA expression level of *Il1b*, *Tnf*, and *Il6* in liver mononuclear cells (MNCs). TG, triglyceride; LPF, low power fields. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4. Discussion

Ginsenosides have been considered a time-honored complementary and alternative medicine for various diseases. The composition of ginsenoside compounds has been identified and classified into PPD-, PPT-, oleanane-, and ocotillo types, which are composed of dammarane backbones with sugar moieties. Recently, as it became known that the absorption and efficacy of minor ginsenosides are better than major ginsenosides, their notable therapeutic roles in diseases have been highlighted despite the small proportion they take in ginsenosides [18]. Especially, GF2 was reported to improve MASLD by reducing the accumulation of fat [23,29,30]. However, little has been studied on how the development of steatosis and inflammation is regulated and which targets of ginsenosides act as ligands.

In hepatocytes, the expression of SREBP1c and FAS, which are indispensable for lipogenesis, has already been reported to be reduced by GF2 [23], but its molecular mechanism remains unclear. In this study, we demonstrated the novel molecular mechanism of GF2 in alleviating HFD-induced MASLD by modulating its key regulator, LXR. First, we revealed the direct binding of GF2 to both LXRα and LXRβ through computational modeling of protein-ligand binding prediction and confirmed this binding of GF2 on reducing LXR activity using LXRE-luciferase assay. In addition, we identified that serine 264 and 278 residues are important for GF2 binding on LXRα and LXRβ, respectively. Second, our study suggests that GF2 specifically decreases the transcription activity of LXRα by maintaining the predominance of corepressor binding to LXRα, but not LXRβ, resulting in reduced expression of LXRα downstream genes. In fact, having confirmed the regulation of LXR by minor ginsenoside compounds (Fig. S1B), GF2 emerged to function as an LXRα-selective antagonist by inducing the reduction of *Srebf1* expression, but not *Abca1*. Consistent with our findings, the functions of LXR isoforms, LXRα and LXRβ, have been thoroughly studied previously. It has been reported that LXRβ plays important roles in controlling HDL-cholesterol levels and stimulating cholesterol efflux

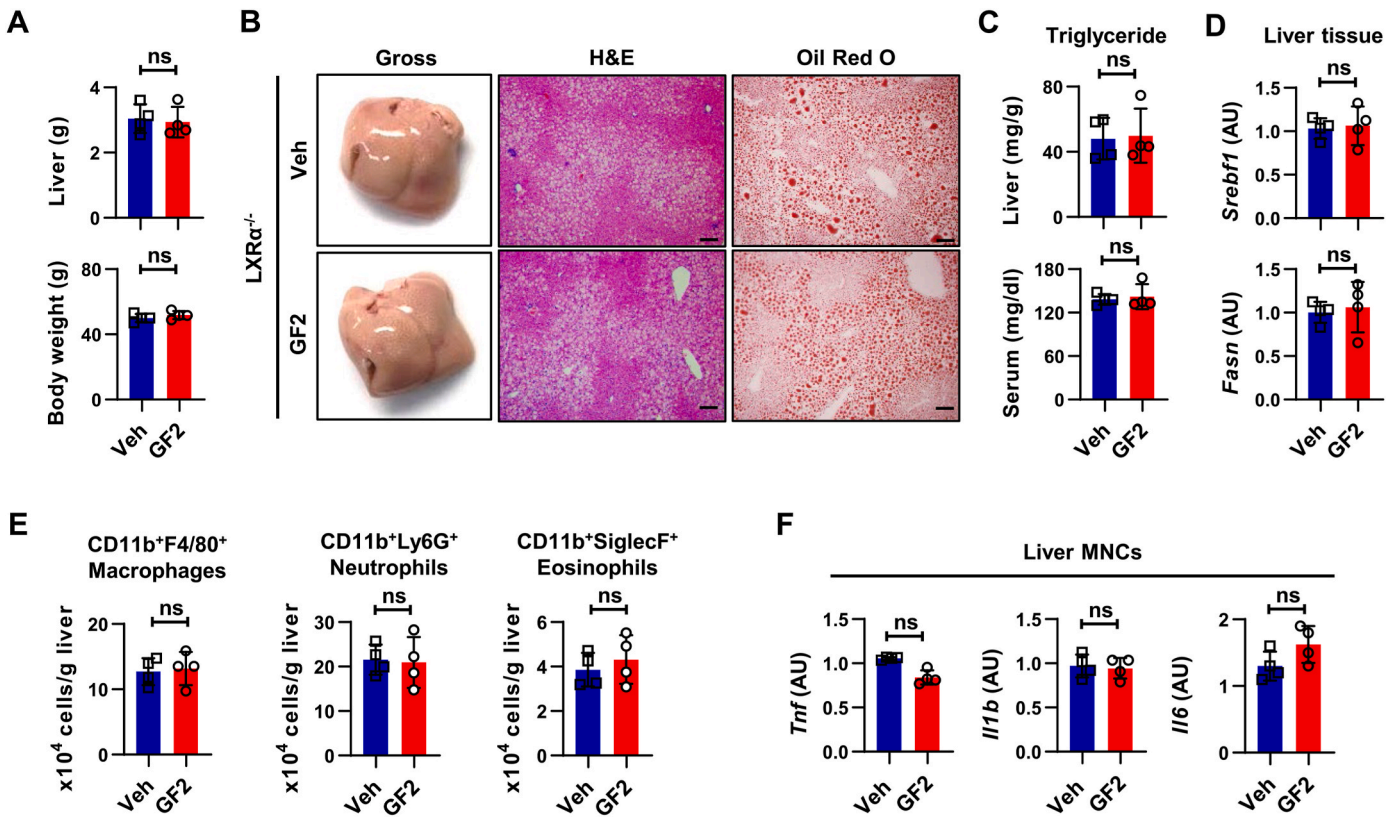

Fig. 6. LXRα deficiency abolishes the effect of GF2 in MASLD.
Physiological and molecular analyses of livers from LXRα$^{-/-}$ mice fed with HFD for 12 weeks with or without GF2 administration (n = 4/group). (A) Body and liver weight of mice. (B) Representative liver gross, H&E, and oil red O staining in liver sections. Scale bar = 100 μm. (C) Hepatic and serum triglyceride levels (D) Relative mRNA expression level of *Srebf1* and *Fasn*. (E) Cell number per g liver of hepatic macrophages, neutrophils, and eosinophils. (F) Relative mRNA expression level of *Il1b, Tnf,* and *Il6* in liver mononuclear cells (MNCs). Data are presented as mean ± SEM. ns, not statistically significant.

in peritoneal macrophages without causing hepatic triglyceride accumulation, whereas deficiency of LXRα/β and LXRα causes a decrease in fat accumulation, suggesting that selective targeting to LXRα without the interference of LXRβ is critical for regulating hepatic steatosis [31]. On the contrary, it was confirmed that ginsenoside Rg1 works as a pan-LXR antagonist by reducing both *Srebf1* and *Abca1*. Accordingly, as our study revealed, GF2 is more efficient than Rg1 in controlling the development of MASLD by specifically regulating LXRα.

LXRs also play crucial roles in the survival, immune response, and differentiation of infiltrated monocytes into macrophages [30,31]. In macrophages, scavenger receptors that are involved in the uptake of oxidized lipoproteins or lipids are critical for the clearance of pathogens, and it is reported that LXR deficiency increases the susceptibility to bacterial infection by impairing the ability of bacterial clearance and inducing apoptosis [32]. Activation of LXR by sterol-like ligands also inhibits the LPS-induced expression of inflammatory genes by interfering with NF-kB signaling in macrophages [33–35]. On the other hand, human pro-inflammatory macrophages highly express LXRs along with downstream genes such as *Abca1*, *Abcg1*, *Lpl,* and *Apoe*. In fact, agonist-induced LXR activation could exacerbate inflammatory response by toll-like receptor-driven cytokine secretion in human macrophages [36], suggesting dual roles of LXRs in pro-inflammatory macrophages. During MASLD development, bone marrow monocytes infiltrate and replenish the dead Kupffer cells by differentiating into monocyte-derived macrophages (MDM) that are more susceptible to inflammatory conditions, posing the possibility of these MDMs expressing LXR with pro-inflammatory activation [37]. However, in our study, GF2 treatment significantly reduced pro-inflammatory cytokine production in both primed WT BMDMs and Kupffer cells, but not in LXRα-deficient cells, suggesting that GF2 may inhibit inflammation through LXRα.

In conclusion, the present study clearly demonstrates that GF2 attenuates MASLD by directly binding with LXRs and sustaining the binding of LXR corepressors, hence reducing the transcription activity of LXRα. This study strongly emphasizes the pharmacological importance of GF2 in the treatment of MASLD by proposing its exact mechanisms and versatile effects.

Author contributions

Kyurae Kim, Myung-Ho Kim, and Won-Il Jeong designed the whole research, Ji In Kang performed ligand binding domain prediction assay and site-directed mutagenesis analysis with advice from Ho Min Kim. Jong-In Baek and Byeong-Min contributed to the preparation of GF2 compound. Kyurae Kim and Won-Il Jeong wrote the manuscript, and Won-Il Jeong and Sun-Chang Kim provided important advice for the experiments.

Declaration of interests

The authors declare that they have no conflicts of interest to disclose.

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Abbreviations

GF2	ginsenoside F2
MASLD	metabolic dysfunction-associated steatotic liver disease
NASH	non-alcoholic steatohepatitis
LXR	liver X receptor
TR-FRET assay	time-resolved fluorescence resonance energy transfer assay
HFD	high-fat diet
LXRE	LXR response element
RXR	retinoid X receptor
SREBP1c	sterol regulatory element binding protein-1c
FASN	fatty acid synthase
MDM	monocyte-derived macrophage
BMDM	bone marrow-derived macrophage
ABCA1	ATP binding cassette subfamily A member 1
LPS	lipopolysaccharide
PPT	protopanaxatriol
PPD	protopanxadiol
ALT	alanine aminotransferase
AST	aspartate aminotransferase
MNC	mononuclear cell

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2023.10.001.

References

[1] Rinella ME, Lazarus JV, Ratziu V, Francque SM, Sanyal AJ, Kanwal F, et al. A multi-society Delphi consensus statement on new fatty liver disease nomenclature. Preprint at Hepatology 2023. https://doi.org/10.1097/HEP.0000000000000520.

[2] Brunt EM, Wong VW, Nobili V, Day CP, Sookoian S, Maher JJ, Bugianesi E, Sirlin CB, Neuschwander-Tetri BA, Rinella ME. Nonalcoholic fatty liver disease. Nat Rev Dis Primers 2015;1:15080.

[3] Riazi K, Azhari H, Charette JH, Underwood FE, King JA, Afshar EE, Swain MG, Congly SE, Kaplan GG, Shaheen AA. The prevalence and incidence of NAFLD worldwide: a systematic review and meta-analysis. Lancet Gastroenterol Hepatol 2022;7(9):851–61.

[4] Bilotta MT, Petillo S, Santoni A, Cippitelli M. Liver X receptors: regulators of cholesterol metabolism, inflammation, autoimmunity, and cancer. Front Immunol 2020;11:584303.

[5] Steffensen KR, Gustafsson JA. Putative metabolic effects of the liver X receptor (LXR). Diabetes 2004;53(Suppl 1):S36–42.

[6] Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, Mangelsdorf DJ. Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. Proc Natl Acad Sci U S A 1999;96(1):266–71.

[7] Calkin AC, Tontonoz P. Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. Nat Rev Mol Cell Biol 2012;13(4):213–24.

[8] Wang B, Tontonoz P. Liver X receptors in lipid signalling and membrane homeostasis. Nat Rev Endocrinol 2018;14(8):452–63.

[9] Pawar A, Botolin D, Mangelsdorf DJ, Jump DB. The role of liver X receptor-alpha in the fatty acid regulation of hepatic gene expression. J Biol Chem 2003;278(42):40736–43.

[10] Higuchi N, Kato M, Shundo Y, Tajiri H, Tanaka M, Yamashita N, Kohjima M, Kotoh K, Nakamuta M, Takayanagi R, et al. Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. Hepatol Res 2008;38(11):1122–9.

[11] Sakai M, Troutman TD, Seidman JS, Ouyang Z, Spann NJ, Abe Y, Ego KM, Bruni CM, Deng Z, Schlachetzki JCM, et al. Liver-derived signals sequentially reprogram myeloid enhancers to initiate and maintain kupffer cell identity. Immunity 2019;51(4):655–670 e658.

[12] N AG, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, Deniz J, Ramirez C, Diaz M, Gallardo G, et al. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. Immunity 2009;31(2):245–58.

[13] N AG, Castrillo A. Liver X receptors as regulators of macrophage inflammatory and metabolic pathways. Biochim Biophys Acta 2011;1812(8):982–94.

[14] Seidman JS, Troutman TD, Sakai M, Gola A, Spann NJ, Bennett H, Bruni CM, Ouyang Z, Li RZ, Sun X, et al. Niche-specific reprogramming of epigenetic landscapes drives myeloid cell diversity in nonalcoholic steatohepatitis. Immunity 2020;52(6):1057–1074 e1057.

[15] Park M, Yoo JH, Lee YS, Park EJ, Lee HJ. Ameliorative effects of black ginseng on nonalcoholic fatty liver disease in free fatty acid-induced HepG2 cells and high-fat/high-fructose diet-fed mice. J Ginseng Res 2020;44(2):350–61.

[16] Ruckdeschel JC. Management of malignant pleural effusion: an overview. Semin Oncol 1988;15(3 Suppl 3):24–8.

[17] Kim MH, Kim HH, Jeong JM, Shim YR, Lee JH, Kim YE, Ryu T, Yang K, Kim KR, Jeon BM, et al. Ginsenoside F2 attenuates chronic-binge ethanol-induced liver injury by increasing regulatory T cells and decreasing Th17 cells. J Ginseng Res 2020;44(6):815–22.

[18] Liu CY, Zhou RX, Sun CK, Jin YH, Yu HS, Zhang TY, Xu LQ, Jin FX. Preparation of minor ginsenosides C-Mc, C-Y, F2, and C-K from American ginseng PPD-ginsenoside using special ginsenosidase type-I from Aspergillus Niger g.848. J Ginseng Res 2015;39(3):221–9.

[19] Shin JY, Lee JM, Shin HS, Park SY, Yang JE, Cho SK, Yi TH. Anti-cancer effect of ginsenoside F2 against glioblastoma multiforme in xenograft model in SD rats. J Ginseng Res 2012;36(1):86–92.

[20] Shin HS, Park SY, Hwang ES, Lee DG, Song HG, Mavlonov GT, Yi TH. The inductive effect of ginsenoside F2 on hair growth by altering the WNT signal pathway in telogen mouse skin. Eur J Pharmacol 2014;730:82–9.

[21] Park SH, Seo W, Eun HS, Kim SY, Jo E, Kim MH, Choi WM, Lee JH, Shim YR, Cui CH, et al. Protective effects of ginsenoside F2 on 12-O-tetradecanoylphorbol-13-acetate-induced skin inflammation in mice. Biochem Biophys Res Commun 2016;478(4):1713–9.

[22] Zhou J, Zhang J, Li J, Guan Y, Shen T, Li F, Li X, Yang X, Hu W. Ginsenoside F2 suppresses adipogenesis in 3T3-L1 cells and obesity in mice via the AMPK pathway. J Agric Food Chem 2021;69(32):9299–312.

[23] Kim DE, Chang BY, Jeon BM, Baek JI, Kim SC, Kim SY. SGL 121 attenuates nonalcoholic fatty liver disease through adjusting lipid metabolism through AMPK signaling pathway. Int J Mol Sci 2020;21(12).

[24] Trott O, Olson AJ, Vina AutoDock. Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 2010;31(2):455–61.

[25] Weiskirchen R, Friedman SL. Hepatic stellate cells : methods and protocols. New York, NY: New York, NY: Humana Press; 2023.

[26] Lu JM, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7(3):293–302.

[27] Kazankov K, Jorgensen SMD, Thomsen KL, Moller HJ, Vilstrup H, George J, Schuppan D, Gronbaek H. The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Nat Rev Gastroenterol Hepatol 2019;16(3):145–59.

[28] Kim SY, Jeong JM, Kim SJ, Seo W, Kim MH, Choi WM, Yoo W, Lee JH, Shim YR, Yi HS, et al. Pro-inflammatory hepatic macrophages generate ROS through NADPH oxidase 2 via endocytosis of monomeric TLR4-MD2 complex. Nat Commun 2017;8(1):2247.

[29] Gu D, Yi H, Jiang K, Fakhar SH, Shi J, He Y, Liu B, Guo Y, Fan X, Li S. Transcriptome analysis reveals the efficacy of ginsenoside-Rg1 in the treatment of nonalcoholic fatty liver disease. Life Sci 2021;267:118986.

[30] Xu Y, Yang C, Zhang S, Li J, Xiao Q, Huang W. Ginsenoside Rg1 protects against non-alcoholic fatty liver disease by ameliorating lipid peroxidation, endoplasmic reticulum stress, and inflammasome activation. Biol Pharm Bull 2018;41(11):1638–44.

[31] Lund EG, Peterson LB, Adams AD, Lam MH, Burton CA, Chin J, Guo Q, Huang S, Latham M, Lopez JC, et al. Different roles of liver X receptor alpha and beta in lipid metabolism: effects of an alpha-selective and a dual agonist in mice deficient in each subtype. Biochem Pharmacol 2006;71(4):453–63.

[32] Joseph SB, Bradley MN, Castrillo A, Bruhn KW, Mak PA, Pei L, Hogenesch J, O'Connell R M, Cheng G, Saez E, et al. LXR-dependent gene expression is important for macrophage survival and the innate immune response. Cell 2004;119(2):299–309.

[33] Spann NJ, Garmire LX, McDonald JG, Myers DS, Milne SB, Shibata N, Reichart D, Fox JN, Shaked I, Heudobler D, et al. Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. Cell 2012;151(1):138–52.

[34] Treuter E. New wrestling rules of anti-inflammatory transrepression by oxysterol receptor LXR revealed. Cell Res 2011;21(5):711–4.

[35] Ito A, Hong C, Rong X, Zhu X, Tarling EJ, Hedde PN, Gratton E, Parks J, Tontonoz P. LXRs link metabolism to inflammation through Abca1-dependent regulation of membrane composition and TLR signaling. Elife 2015;4:e08009.

[36] Gonzalez de la Aleja A, Herrero C, Torres-Torresano M, de la Rosa JV, Alonso B, Capa-Sardon E, Muller IB, Jansen G, Puig-Kroger A, Vega MA, et al. Activation of LXR nuclear receptors impairs the anti-inflammatory gene and functional profile of M-CSF-dependent human monocyte-derived macrophages. Front Immunol 2022;13:835478.

[37] Wen Y, Lambrecht J, Ju C, Tacke F. Hepatic macrophages in liver homeostasis and diseases-diversity, plasticity and therapeutic opportunities. Cell Mol Immunol 2021;18(1):45–56.