Supplementary Information for

Oral administration of PEGylated TLR7 ligand ameliorates alcohol-associated liver disease via the induction of IL-22.

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Supplementary Methods

Alanine aminotransferase and hepatic triglyceride measurement

Whole-blood samples were collected from mice via cardiac puncture and were left at room temperature for 30 min. The clot was removed by centrifugation at 5,000 rpm for 15 min. Serum alanine aminotransferase (ALT) concentrations were determined by Infinity ALT (GPT) Liquid Stable Reagent (#TR71121, Thermo Scientific). Hepatic triglycerides (TGs) levels were measured using a triglyceride measurement kit (Pionte Scientific).

RNA isolation and quantitative real-time PCR analysis

RNA was extracted from primary cells and tissues of the liver and small intestines using TRIzol (Life Technologies) plus NucleoSpin (Clontech). Extracted RNA was converted to complementary DNA using a reverse transcription kit (Applied Biosystems). Quantitative real-time PCR was performed using SYBR Green. Quantification was performed by comparing sample Ct values after normalization to 18S RNA levels. Primer sequences are summarized in Table S1.

Primary cell isolation

Kupffer cells, pDCs, B cells, and hepatic stellate cells were isolated from mice by *in situ* liver perfusion with pronase E (#10165921001, Sigma-Aldrich) and collagenase D (#11088882001, Sigma-Aldrich), followed by density gradient centrifugation with Nycodenz (#AN1002424, Accurate Chemical and Scientific Corporation), as previously described(1). Magnetic antibody sorting (MACS; Miltenyi Biotec) using CD11b (#130-097-142, Miltenyi Biotec), BST2 (#130-107-093, Miltenyi Biotec), and B220 antibodies (#130-097-152, Miltenyi Biotec) was performed to separate Kupffer cells, pDCs, and B cells, respectively. The purity of hepatic stellate cells was evaluated by vitamin A autofluorescence. Primary hepatocytes were obtained by collagenase perfusion, as previously described (1). The trypan blue exclusion assay was used to confirm 90% or greater cell viability.

For isolation of lamina propria lymphocytes (LPLs)

Small intestines were harvested and placed in ice-cold phosphate-buffered saline (PBS). After removing feces by flushing with ice-cold PBS, intestinal tissues were opened and cut. Tissues were then incubated in Hank's Balanced Salt Solution (HBSS) with 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mg/mL collagenase IV. Cells were washed, filtered, and centrifuged using Percoll gradient centrifugation (2).

FACS analysis

LPLs were treated with Brefeldin A solution (eBioscience) and then labeled with fluorescence tagged antibodies; under the anti-mouse CD16/CD32 (mouse Fc blocker, Clone 2.4G2; BioLegend) and the Live/dead Ghost Dye™ UV450 (Tonbo Bioscience). IL-22 producing lymphocytes were stained by PE-conjugated anti-mouse IL-22 (Clone Poly5164; BioLegend), FITC-conjugated CD3ε (Clone 145-2D11; BioLegend), PerCP-Cy5.5-conjugated CD4 (Clone RM4-5; BioLegend), and RORγt (clone AFKJS-9; eBioscience). IL-22 and RORγt antibodies were

applied under fixation/permeabilization procedure (eBioscience). Stained cells read with FACS LSR II (BD Biosciences), and the result was analyzed by FlowJo software (BD/Flow Jo LLC). Single cells were filtered by FSC-A/H/W, and dead cells were removed by GhostDye^{TM} UV450. From these gating, live CD45$^+$ IL-22$^+$ Lymphocytes were analyzed through pseudo-color analysis plot. IL-22 producing cells were determined by anti-IL-22, and those cells were further analyzed by anti-CD3, CD4, and RORγt. RORγt$^+$ cells were separated by anti-CD3 and CD4 for comparison between vehicle and 1Z1 treatment group.

Cell culture experiments

Kupffer cells were maintained in RPMI medium with 10% fetal bovine serum (FBS) for 6 h and were then serum deprived overnight. The next day, the cells were treated with R848 (0.5 µM) and 1Z1 (5 µM) for 6 h. Kupffer cells were also pre-treated with CpG-DNA (5 µg/mL) for 16 h, followed by treatment with 1Z1 (5 µM) for an additional 6 h. For some experiments, Kupffer cells and pDCs were treated with miR-21a and miR-29a (100 nM) for 4 h.

Extracellular Vesicles (EV) Isolation

For EV isolation from cell culture medium, cells were cultured in DMEM with exosome-depleted Fetal Bovine Serum (Cat.No. A2720801; Thermo Scientific) and treated with ethanol (100 mM) for 24 hours. The medium was centrifuged at 300×g for 10min to remove the whole cells and followed by 2000×g centrifugation for 10min to remove the debris and apoptotic bodies. The resulting medium was applied to a 0.22µm vacuum filtration unit (Cat.No: S2GPU05RE; Sigma Aldrich) to eliminate large EVs and then centrifuged at 100000×g for 70mins. Pellets were resuspended in PBS and centrifuged a second time at 100000×g for 70mins. The final pellet was resuspended in PBS. The protein concentration of the EV samples was evaluated by the BCA kit (Cat,No:23225; Thermo Scientific). For EV isolation from mouse serum, frozen mouse serum was thawed in a 37°C water bath and applied to a 0.22µm filter to remove large EVs. EV isolation was performed on the ExoQuick(R) ULTRA EV Isolation Kit for Serum(Cat No: EQULTRA-20A-1; System Biosciences) according to the instructions. The resulting pellets were resuspended in PBS.

High-throughput sequencing of 16S rDNA and data analysis

Mouse fecal samples were sterilely collected and stored at -80°C. DNA was isolated from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen), according to the manufacturer's protocol.

The 16S rRNA was amplified from the extracted DNA using the following primers: 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and R357 (5'-CTGCTGCCTYCCGTA-3'). The PCR products were purified using HighPrep PCR Magnetic Beads (MagBio Genomics) and analyzed using the DNA 1000 assay on an Agilent 2100 Bioanalyzer System (Agilent Technologies). Amplicons were then uniquely indexed in each PCR reaction using Nextera XT Index Kit v2 Sets A–D (Illumina) and KAPA HiFi HotStart ReadyMix (Roche). Library enrichment was performed using 10 PCR cycles, and amplicons were purified using Agencourt Ampure Magnetic Beads (Beckman). Multiplexed samples were pooled at equal volumes according to library type. The pooled libraries were purified using HighPrep PCR Magnetic Beads and subsequently assayed on an Agilent 2100 Bioanalyzer to confirm the final sizes. Samples were sequenced on the MiSeq platform (Illumina) with a paired-end 300-bp sequencing chemistry. Raw data processing and run

demultiplexing were performed using on-instrument analytics, according to the manufacturer's recommendations.

Raw FASTQ data were merged with overlap into single reads using SeqPrep v1.0 wrapped by QIIME v1.9 with the default setting (3). A custom script was used to remove any reads that did not contain the proximal primer sequence or that contained a single unknown base (N) for enrichment of high quality reads. The remaining high quality reads were aligned to the Greengenes reference database (May 2013 release) using BLAST v2.2.22 in QIIME v1.9 wrapper with an identity percentage ≥97% for the selection of operational taxonomic units (OTUs). An OTU table was generated and included the number of sequence reads that represented each OTU, along with the taxonomic classification.

A web-based tool, MicrobiomeAnalyst (http://www.microbiomeanalyst.ca/), was used for further statistical analysis and data visualization (4,5). The OTU table with the taxa in plain format and the metadata file were uploaded to the MicrobiomeAnalyst tool. Data were filtered using the default options and unclassified reads were excluded. Alpha diversity was analyzed based on the Chao1 index, and beta diversity was analyzed based on the weighted UniFrac distance metric.

Supplementary References

1. Y. S. Roh, B. Zhang, R. Loomba, E. Seki, TLR2 and TLR9 contribute to alcohol-mediated liver injury through induction of CXCL1 and neutrophil infiltration. *Am J Physiol Gastrointest Liver Physiol* **309**, G30-41 (2015).
2. T. Hendrikx *et al.*, Bacteria engineered to produce IL-22 in intestine induce expression of REG3G to reduce ethanol-induced liver disease in mice. *Gut* **68**, 1504-1515 (2019).
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5. A. Dhariwal *et al.*, MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res* **45**, W180-W188 (2017).

Supplementary Figures

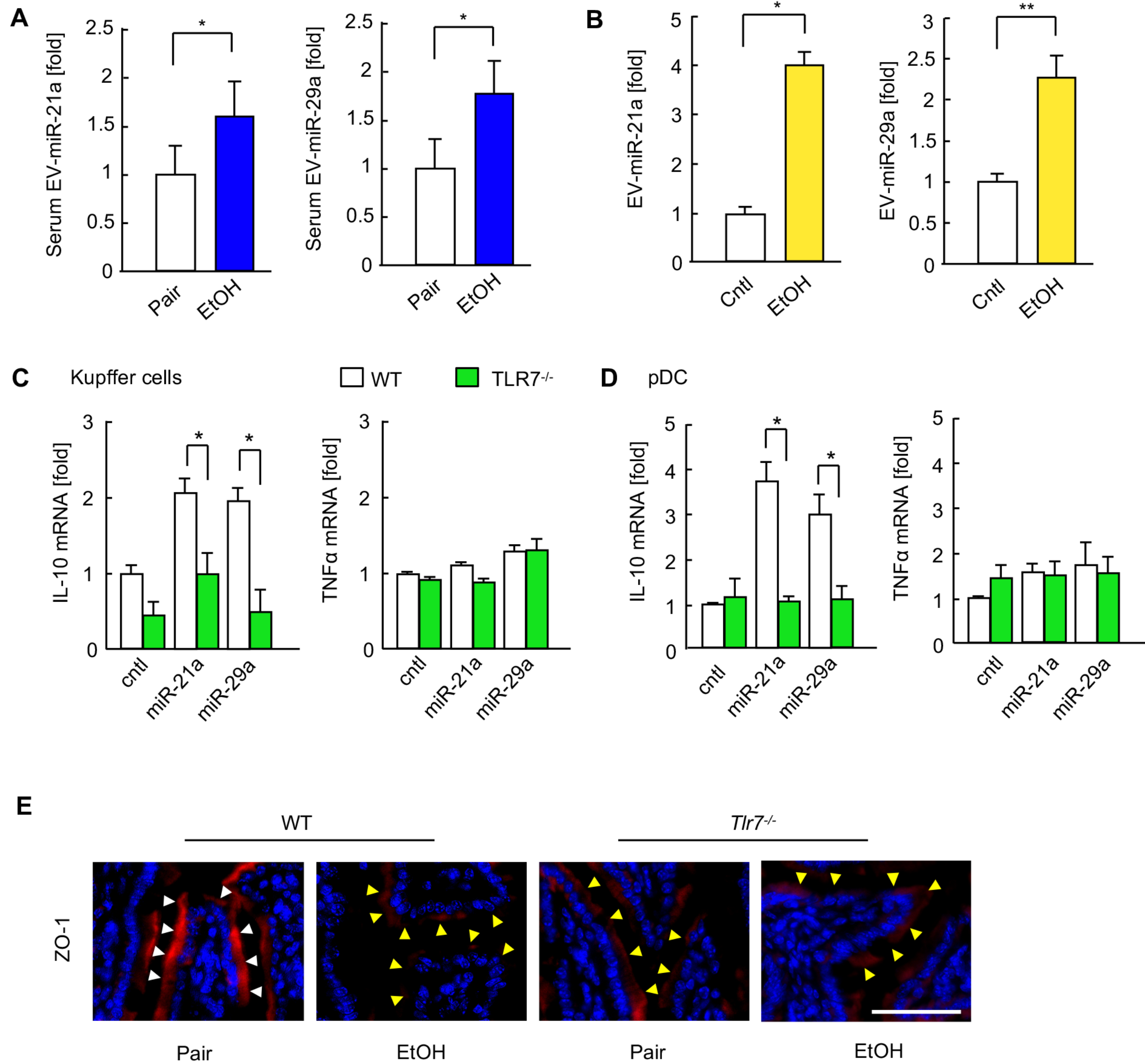

Fig. S1. MicroRNAs as endogenous ligands for TLR7 and intestinal ZO-1 expression in WT and *Tlr7*$^{-/-}$ mice.

(***A,B***) Levels of miR-21a and miR-29a in extracellular vesicles (EVs) from serum of mice with pair- and ethanol-containing diet feeding (n=7-8/group) (***A***), and from culture media of vehicle- and ethanol (100mM)-treated hepatocytes (n=5, each) (***B***). (***C,D***) IL-10 and TNFα mRNA expression in Kupffer cells (***C***) and plasmacytoid dendritic cells (pDCs) (***D***) treated with scramble, miR-21a or miR-29a (100nM) for 4 hours. *P<0.05, **P<0.01. Data are mean±s.e.m. Two-tailed Student's *t*-test (***A,B***) and one-way ANOVA with Tukey's *post hoc* analysis (***C,D***). (***E***) Representative immunofluorescence staining images for ZO-1 in the small intestines. White arrowhead, maintained ZO-1 expression; Yellow arrowhead, decreased ZO-1 expression. Scale bar 50 μm.

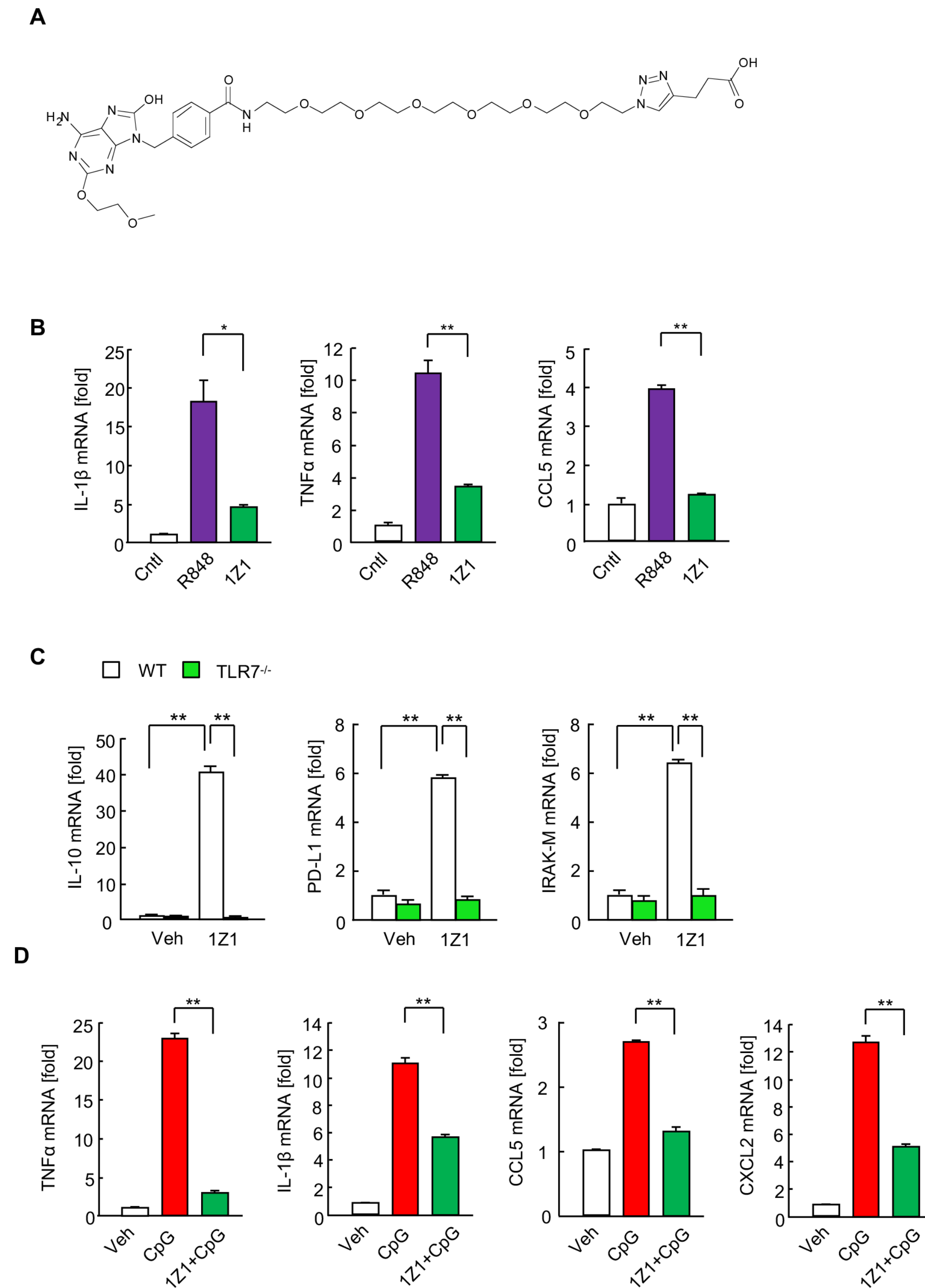

Fig. S2. Response of Kupffer cells to 1Z1 *in vitro*.

(***A***) The chemical structure of 1Z1. (***B***) IL-1β, TNFα, and CCL5 mRNA expression in Kupffer cells treated with R848 (0.5 μM) and 1Z1 (5 μM) for 6 h. (***C***) IL-10, PD-L1, and IRAK-M mRNA expression in Kupffer cells treated with 1Z1 (5 μM) for 6 h. (***D***) TNFα, IL-1β, CCL5, and CXCL2 mRNA expression in Kupffer cells pre-treated with CpG-DNA (5 μg/mL) for 16 h, followed by treatment with 1Z1 (5 μM) for an additional 6 h. **P*<0.05, ***P*<0.01. Data are presented as the mean ± SEM One-way ANOVA with Tukey's *post hoc* analysis.

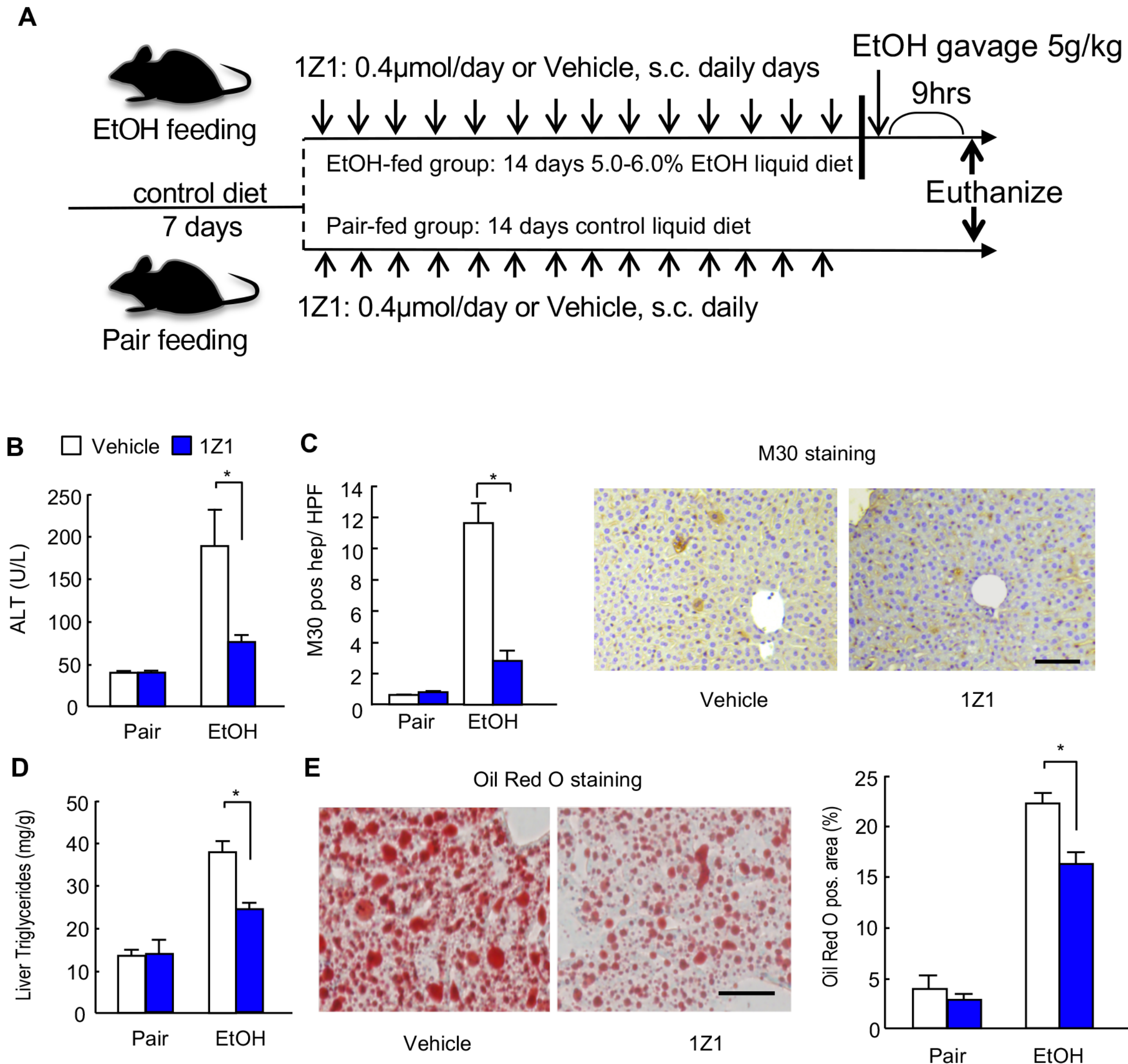

Fig. S3. Treatment schedule and the findings of subcutaneous treatment of 1Z1 in the chronic-binge ethanol-feeding model.

(***A***) The schedule for ethanol feeding and subcutaneous 1Z1 treatment. (***B***) Serum ALT levels. (***C***) Quantification of M30-positive hepatocytes (left). Representative images of M30 staining (right). (***D***) Liver triglycerides levels. (***E***) Representative images of oil red O staining (left). Quantification of oil red O-positive area (right). Data are mean±s.e.m. **P*<0.05. One-way ANOVA with Tukey's *post hoc* analysis. s.c., subcutaneous. Scale bar 50 μm.

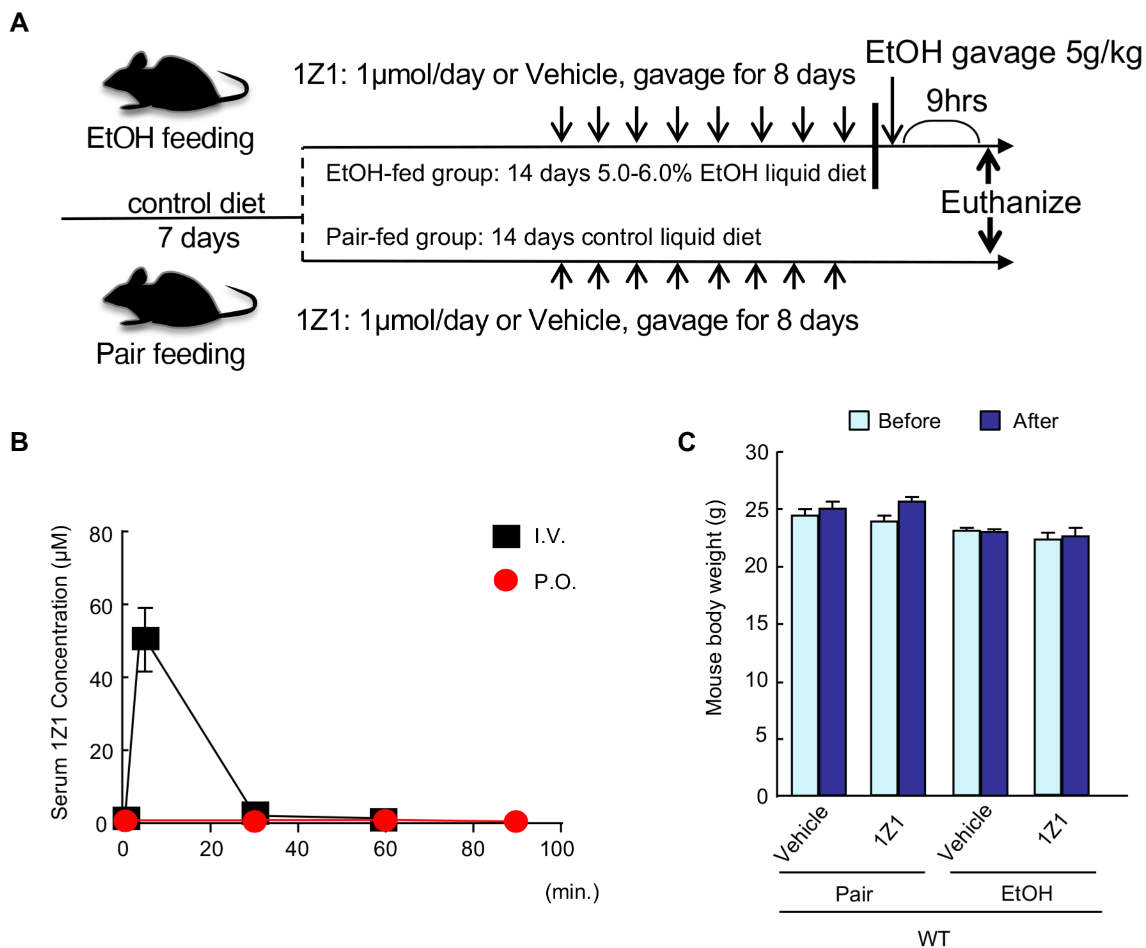

Fig. S4. Schedule of oral 1Z1 treatment in the chronic-binge ethanol-feeding model.

(***A***) The schedule for ethanol feeding and oral 1Z1 treatment. (***B***) The pharmacokinetics analysis is shown. Serum 1Z1 concentrations were measured after intravenous injection of 0.4 µM or oral gavage of 1 µM of 1Z1 after 1 week of feeding of ethanol-containing diet. (***C***) Mouse body weight was measured before and after ethanol feeding and/or 1Z1 treatment.

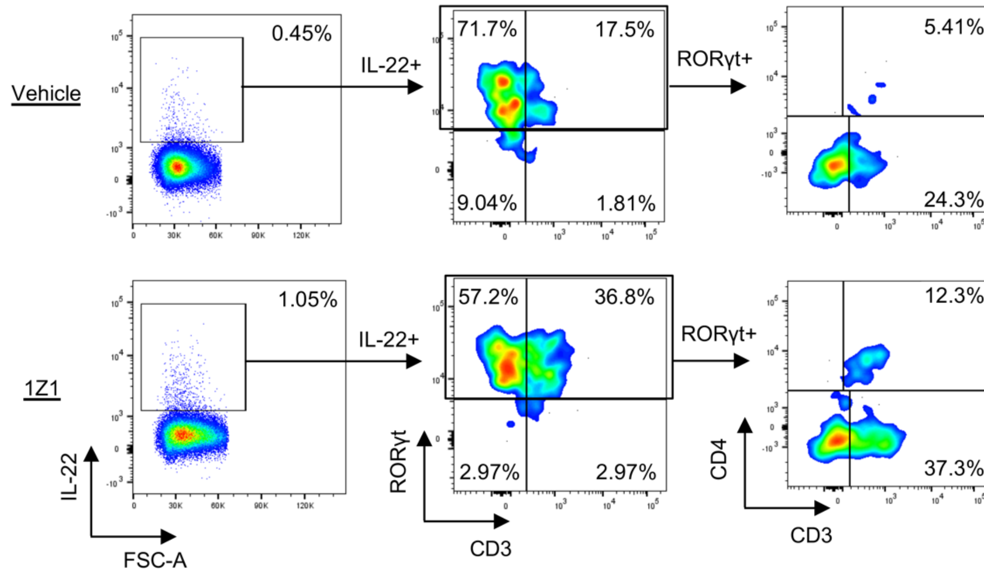

Fig. S5. 1Z1-mediated IL-22 producing cells in lamina propria lymphocytes.

Lamina propria cells were isolated from oral vehicle or 1Z1-treated mice. Cells were stained for CD3, CD4, intracellular IL-22 and RORγt.

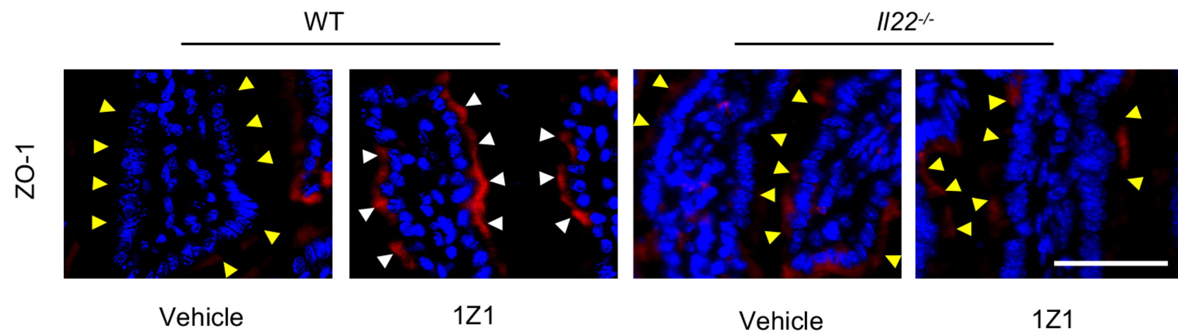

Fig. S6. Immunofluorescence of intestinal ZO-1 expression in WT and *Il22*$^{-/-}$ mice.

Representative immunofluorescence staining images for ZO-1 in the small intestines. White arrowhead, maintained ZO-1 expression; Yellow arrowhead, decreased ZO-1 expression. Scale bar 50 μm.

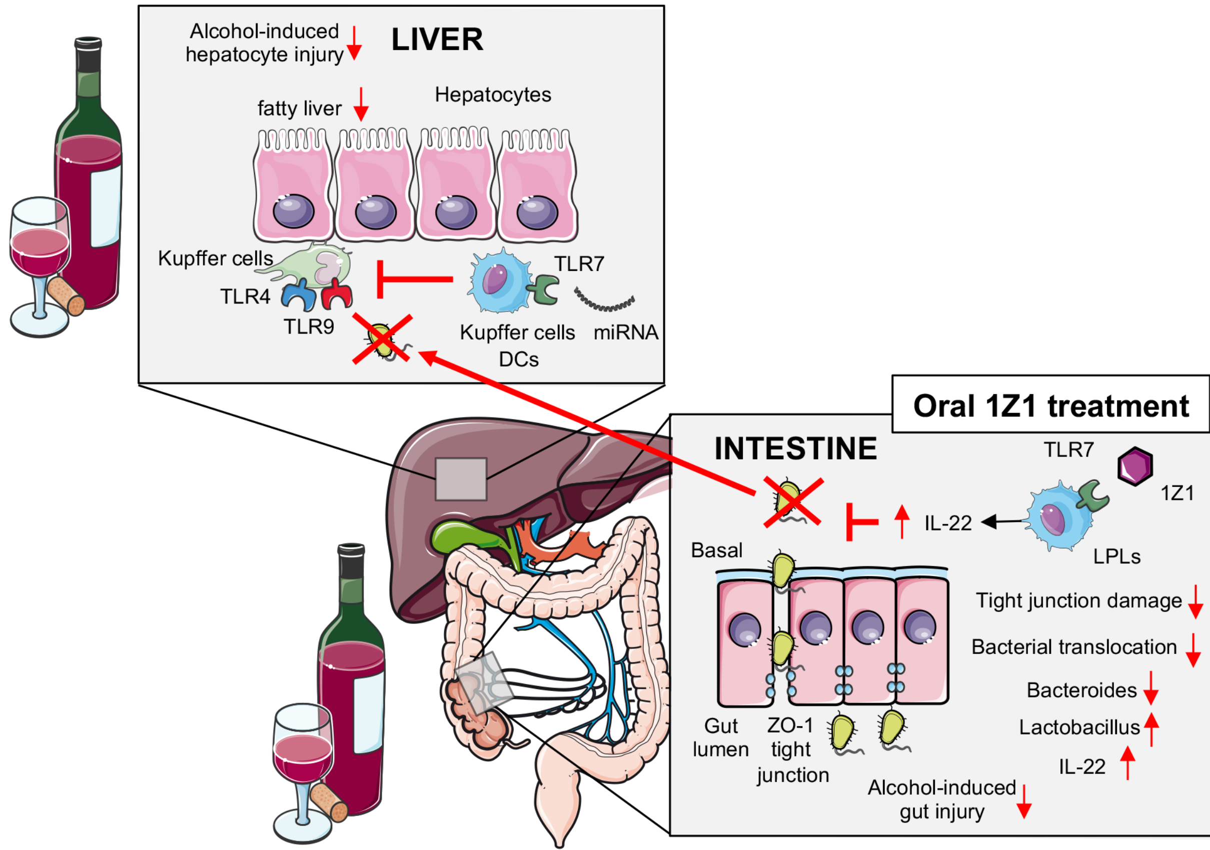

Fig. S7. The proposed mechanism action of 1Z1 for treating AH through intestinal IL-22 induction.

Oral 1Z1 administration induces intestinal IL-22 expression, which protects against ethanol-induced intestinal tight junction damage and bacterial translocation, resulting in the protection of ethanol-induced liver damage.

Supplementary Table 1 Sequence of Primers Used for Real-Time Quantitative PCR

18s	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA
TLR7	ATGTGGACACGGAAGAGACAA	GGTAAGGGTAAGATTGGTGGTG
TNFα	AGGGTCTGGGCCATAGAACT	CCACCACGCTCTTCTGTCTAC
IL-1β	GGTCAAAGGTTTGGAAGCAG	TGTGAAATGCCACCTTTTGA
IL-6	ACCAGAGGAAATTTTCAATAGGC	TGATGCACTTGCAGAAAACA
IL-10	TGT CAA ATT CAT TCA TGG CCT	ATC GAT TTC TCC CCT GTG AA
IL-22	ATGAGTTTTTCCCTTATGGGGAC	GCTGGAAGTTGGACACCTCAA
CXCL2	AAAGTTTGCCTTGACCCTGAA	CTCAGACAGCGAGGCACATC
CCL5	CCACTTCTTCTCTGGGTTGG	GTGCCCACGTCAAGGAGTAT
IRAK-M	CCAGCCAGCTGTTTGAAAGT	CACTGCTGGGAGAGCTTTG
PD-L1	CCACGGAAATTCTCTGGTTG	TGCTGCATAATCAGCTACGG
ZO-1	CCTGTGAAGCGTCACTGTGT	CGCGGAGAGAGACAAGATGT
Reg3b	ACTCCCTGAAGAATATACCCTCC	CGCTATTGAGCACAGATACGAG
Reg3g	ATGCTTCCCCGTATAACCATCA	GGCCATATCTGCATCATACCAG
α-defensin	GGTGATCATCAGACCCCAGCATCAGT	AAGAGACTAAAACTGAGGA GCAGC