**Irf5 siRNA-loaded biodegradable lipid nanoparticles ameliorate concanavalin A-induced liver injury**

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INTRODUCTION

RNA interference-based gene silencing drugs are attracting attention for treating various diseases. Lipid nanoparticles (LNPs) are carriers that efficiently deliver small interfering RNA (siRNA) to the cytoplasm of target cells. Recently, we developed potent and well-tolerated biodegradable LNPs with asymmetric ionizable lipids. Here, we evaluated the effect of LNPs on immune cells in mice. After intravenous administration, LNPs were efficiently incorporated into several tissue-resident macrophages, including liver macrophages, through an apolipoprotein E (ApoE)-independent mechanism. Administration of LNP-encapsulated siRNA against Irf5, encoding the transcription factor critical for inflammatory responses, sharply reduced its expression in macrophages in vivo, and persisted for as long as 7 days. The therapeutic potential of Irf5 siRNA-loaded LNPs in inflammatory diseases was tested in a concanavalin A (Con A)-induced hepatitis model, whose pathogenic mechanisms are dependent on cytokine secretion from macrophages. We found that Con A-induced liver injury was significantly attenuated after LNP injection. Serum aspartate transaminase, alanine aminotransferase, and inflammatory cytokine levels were significantly reduced in mice injected with Irf5 siRNA-loaded LNPs compared to those injected with control siRNA-loaded LNPs. Our results suggest that administering biodegradable LNPs to deliver siRNA is a promising strategy for treating inflammatory disorders.

In 2018, the first siRNA-based medicine was approved by the United States Food and Drug Administration.5,6 This drug utilizes lipid nanoparticles (LNPs) as the siRNA carrier. LNPs efficiently deliver siRNA to the cytoplasm and are mainly composed of ionizable lipids, phospholipids, cholesterol, and polyethylene glycol (PEG) lipids that protect siRNA from nucleases.7,8 The characteristics of LNPs are largely dependent on the types of ionizable lipids.7,8 Ionizable lipids are critical for endosomal disruption and siRNA release from LNPs in target cells,9 but they can cause cytoxicity due to non-specific binding to proteins.7 There are various types of ionizable lipids, such as symmetric ionizable lipids,10 asymmetric ionizable lipids,11 and lipidoids.12 Among them, asymmetric ionizable lipids effectively lead to target gene silencing and are less cytoxic than other ionizable lipids.11,13,14 Recently, we developed biodegradable LNPs comprised of novel ionizable lipids, L120, with an asymmetric lipid tail.12,17 The ester bond in the ionizable lipid tail is hydrolyzed in vivo, leading to rapid lipid degradation and reduced cytoxicity. We have shown that our biodegradable LNPs were successfully incorporated into liver hepatocytes and led to effective knockdown of target mRNA expression.12

Apolipoprotein E (ApoE) and low-density lipoprotein receptor (LDLR) are involved in the incorporation of LNPs into hepatocytes in vivo.16 Plasma ApoE preferentially binds to PEG lipids of the

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Mononuclear phagocytes, including macrophages and monocytes, down-regulate and then observe localization of AF488-labeled siRNA using fluorescence microscopy. Nuclei were stained with DAPI. Data are representative of two independent experiments with similar results. Scale bars represent 10 μm. cDC, classical dendritic cell; pDC, plasmacytoid dendritic cell; Mo, monocyte; Mφ, macrophage; Neu, neutrophil; Eos, eosinophil; Baso, basophil; MFI, mean fluorescence intensity. See also Figures S1, S2, and S3.

Figure 1. Incorporation of biodegradable LNPs into immune cells

(A–C) Flow cytometry analysis of AF488-labeled siRNA distribution following intravenous injection of LNPs in mice. Immune cells in the spleen (A), peritoneal exudate cells (B), and liver (C) were analyzed 3 h after intravenous injection of LNPs containing AF488-labeled siRNA at a siRNA dose of 0.8 mg/kg. The MFI of AF488 in each immune cell population is shown in the boxplots. Values from two independent experiments are shown. A representative dot plot of liver macrophage fluorescence signal of biodegradable LNPs was most abundant in splenic macrophages, and to a lesser degree in monocytes 3 h after LNP injection. B Bone marrow hematopoietic stem and progenitor cells, splenic lymphocytes, dendritic cells, neutrophils, eosinophils, and basophils did not become cationic due to reduced pH during endosome maturation, resulting in LNP incorporation via endocytosis. LNPs were strongly incorporated into tissue-resident macrophages, including liver macrophages. Analysis of ApoE-deficient mice revealed that LNP uptake by liver macrophages is not dependent on ApoE expression. Administering LNPs encapsulating siRNA targeting interferon regulatory factor-5 (IRF5) (siIRF5-LNPs), an essential transcription factor in triggering inflammatory responses, reduced IRF5 expression in macrophages in vivo. Furthermore, prophylactic administration of siIRF5-LNPs was effective in preventing liver damage in concanavalin A (Con A)-induced hepatitis. These results indicate that our biodegradable LNPs are a useful tool for analyzing macrophage function in vivo, and they can be used in novel therapies for treating inflammatory diseases.

RESULTS

Biodegradable LNPs are mainly incorporated by macrophages in immune cell populations

To examine the in vivo distribution of biodegradable LNPs in immune cells, we administered biodegradable LNPs containing Alexa Fluor 488 (AF488)-labeled siRNA into mice through intravenous injections and analyzed bone marrow cells and splenocytes (Figure S1). Flow cytometry analysis revealed that the fluorescence signal of AF488-labeled siRNA was most abundant in splenic macrophages, and to a lesser degree in monocytes 3 h after LNP injection. Bone marrow hematopoietic stem and progenitor cells, splenic lymphocytes, dendritic cells, neutrophils, eosinophils, and basophils did not effectively take up the LNPs (Figures 1A, S2, and S3A).

Next, we investigated the distribution of biodegradable LNPs in peritoneal and liver immune cells. Three hours after intravenous administration of LNPs, peritoneal exudate cells were collected and analyzed by flow cytometry. We found that the biodegradable LNPs were taken up by peritoneal macrophages, but not by B lymphocytes (Figures 1B and S3B). There are several macrophage and monocyte subpopulations in the liver. We confirmed that cells expressing F4/80, a typical marker for macrophages, were mainly composed of F4/80CD11b+ cells (gated in R1), F4/80CD11b low cells (R2), and F4/80CD11b high cells (R3) (Figure 1C). Previous studies suggested that the F4/80 CD11b+ population corresponds to monocytes and monocyte-derived macrophages, while F4/80CD11b low and F4/80CD11b high populations mainly correspond to liver-resident Kupffer cells. Among the three F4/80-expressing populations, biodegradable LNPs were mainly detected in F4/80CD11b+ cells. The uptake of LNPs in F4/80CD11b+ cells, F4/80CD11b high cells, and B cells was less prominent (Figures 1C and S3C). To assess whether siRNAs in LNPs were incorporated into the cytoplasm of target cells, we isolated liver F4/80CD11b+ macrophages and B cells after injection of LNPs and then observed localization of AF488-labeled siRNA using...
confocal microscopy. As expected, AF488-labeled siRNA was present in the cytoplasm of liver F4/80^{-}\text{CD11b}^{+} macrophages, but not in B cells (Figure 1D). These results suggest that the biodegradable LNPs are efficiently incorporated into resident macrophage populations, such as liver F4/80^{-}\text{CD11b}^{+} macrophages. Notably, we previously compared the uptake of our LNPs between liver macrophages and hepatocytes. The rates of LNPs incorporated by macrophages and hepatocytes were approximately 40\% and 60\%, respectively,\textsuperscript{15} indicating that most LNPs are taken up by hepatocytes in the liver.

**Biodegradable LNP administration induces knockdown of a target molecule in macrophages in vivo**

To assess the gene silencing effect of biodegradable LNPs in macrophages, we selected interferon regulatory factor-5 (IRF5) as a target molecule. The transcription factor IRF5 is strongly expressed in macrophages, monocytes, dendritic cells, and B cells.\textsuperscript{32,33} Pathogen-associated molecular patterns such as single-stranded RNA and CpG DNA activate IRF5 via Toll-like receptor (TLR)-mediated signaling.\textsuperscript{34,35} Activated IRF5 translocates from the cytoplasm to the nucleus, leading to expression of type I interferons and proinflammatory cytokines such as tumor necrosis factor (TNF)-\textalpha and interleukin (IL)-6.\textsuperscript{34,35} To investigate the therapeutic effect of the biodegradable LNPs in inflammatory disorders, we generated LNPs containing siRNA against Irf5 mRNA (siIrf5-LNPs) (see Figure S1).

We first determined the optimal dosage of siIrf5-LNPs in vivo. The data indicated that the effect of suppressing Irf5 expression in peritoneal macrophages reached a plateau at a dose of 5.0 mg/kg (Figures 2A and S4). Irf5 mRNA expression was also downregulated significantly in splenic and liver macrophages (Figure 2A). In contrast, Irf5 mRNA expression in B cells was not affected by siIrf5-LNP administration. No Irf genes other than Irf5 were suppressed in liver macrophages by the siIrf5-LNPs, indicating the specific knockdown of Irf5 (Figure S5). Consistent with quantitative reverse transcriptase PCR (qRT-PCR) data, IRF5 protein expression in liver F4/80^{-}\text{CD11b}^{+} macrophages was significantly reduced 1, 2, and 7 days after administration (Figure 2B). These results suggest that administration of biodegradable LNPs can induce long-term gene silencing in tissue-resident macrophages in vivo. Notably, Irf5 expression in non-immune liver CD45^{-} cells, possibly including hepatocytes, sinusoidal endothelial cells, and stellate cells, was low and not significantly downregulated by siIrf5-LNP administration (Figure S6).

**LNPs are incorporated into macrophages via an ApoE-independent mechanism**

Previous reports have suggested that biodegradable LNPs are incorporated into hepatocytes via the ApoE-LDLR pathway.\textsuperscript{15,36} To investigate whether the ApoE-LDLR pathway is involved in incorporating biodegradable LNPs used in this study into macrophages, we injected them into ApoE-deficient mice. ApoE-deficient F4/80^{-}\text{CD11b}^{+} macrophages incorporated the LNPs encapsulating AF488-labeled siRNA as much as did wild-type macrophages (Figure 3A). In addition, we found that Irf5 mRNA expression in ApoE-deficient macrophages, but not B cells, was significantly reduced after administration of siIrf5-LNPs (Figure 3B). These results indicate that ApoE is not involved in the uptake of biodegradable LNPs in liver F4/80^{-}\text{CD11b}^{+} macrophages.

**Con A-induced hepatitis is attenuated in Irf5-deficient mice**

The above results revealed that Irf5 expression in liver macrophages can be efficiently downregulated upon injection of biodegradable LNPs. Therefore, we decided to use a Con A-induced hepatitis model, in which liver macrophages play a critical role in pathogenesis. Con A administered through intravenous injection binds to and activates liver macrophages.\textsuperscript{37,38} Subsequently, these cells produce inflammatory cytokines and chemokines, such as TNF-\textalpha and CXCL2, leading to the activation of T cells and natural killer (NK) T cells, and recruitment of neutrophils to the liver.\textsuperscript{40-43} Liver necrosis is eventually caused by these inflammatory cytokines and by direct cytotoxic effects such as Fas-mediated cell death.\textsuperscript{44-46}
To evaluate the validity of targeting IRF5 in this model, we injected Con A into IRF5-deficient mice. As expected, Con A administration induced a significant increase in blood aspartate transaminase (AST) and alanine aminotransferase (ALT) levels, as well as hepatocyte necrosis, in wild-type mice (Figures 4A and 4B). We found that levels of AST and ALT, as well as liver damage, were significantly ameliorated in IRF5-deficient mice. Furthermore, the production of TNF-α, an essential cytokine involved in the pathogenesis of Con A-induced hepatitis, was significantly reduced in IRF5-deficient mice compared to wild-type mice (Figure 4C). To our knowledge, this is the first demonstration that IRF5 is a critical regulator of Con A-induced hepatitis progression. These results suggest that IRF5 is a candidate therapeutic target in this model.

Con A-induced hepatitis is ameliorated by siirf5-LNP administration

To investigate the prophylactic effect of siirf5-LNPs, we administered LNPs twice and then challenged the mice with Con A 4 days after LNP injection (Figure 5A). We found that AST and ALT levels were significantly reduced in these mice compared to control LNP-treated mice (Figure 5B). Consistent with these results, liver necrosis and bleeding were suppressed by siirf5-LNP treatment (Figure 5C). Furthermore, production of TNF-α and IL-6 was significantly downregulated in mice injected with siirf5-LNPs (Figure 5D). Taken together, our results indicate that siirf5-LNP administration reduces IRF5 expression in macrophages, thereby suppressing production of pro-inflammatory cytokines and liver damage after Con A injection.

**DISCUSSION**

LNPs have been primarily used to target hepatocytes in mouse disease models and clinical trials. In this study, we investigated the effects of biodegradable LNPs on immune cells, as well as their potential application in preventing inflammatory diseases. Biodegradable LNPs were strongly incorporated by tissue-resident macrophages, especially liver macrophages. LNP uptake was also observed in ApoE-deficient macrophages, suggesting that LNPs are incorporated through a mechanism independent of the ApoE-LDLR pathway. We also showed that IRF5 is essential for pathogenesis in Con A-induced hepatitis. Furthermore, silirf5-LNP injection resulted in long-term downregulation of IRF5 in liver macrophages and prevented inflammatory cytokine production and liver damage in the Con A-induced hepatitis model.

The molecular mechanisms underlying uptake of biodegradable LNPs into macrophages remain unclear. Previous studies have shown that incorporation of LNPs with symmetric ionizable lipids and lipoids into macrophages is mediated by phagocytosis, but not micropinocytosis or endocytosis. Binding of plasma proteins, such as complement and immunoglobulins, to LNPs can also assist phagocytosis by macrophages in vitro. These findings imply that our biodegradable LNPs are incorporated via phagocytosis in tissue-resident macrophages.

A time-course analysis revealed that knockdown effects of the biodegradable LNPs in liver macrophages persisted for as long as 7 days. Previous reports suggested that most tissue-resident macrophages, including liver Kupffer cells, are generated from fetal macrophage progenitors during embryogenesis. Fetal progenitor-derived liver macrophages are not replenished by bone marrow cells, such as monocytes, and they maintain their numbers by slow proliferation in adult mice. Therefore, siRNA incorporated in macrophages may be sustained due to slow cell division. Likewise, the knockdown effects of LNPs persist longer in hepatocytes, which barely proliferate in steady-state conditions, compared to fast-growing cancer cells. Long-term knockdown by biodegradable LNPs will enable us to analyze the function of target molecules in tissue-resident macrophages in vivo.

The molecular basis for IRF5 activation in Con A-induced hepatitis remains unclear. IRF5 is activated by TLR-MyD88 signaling pathways. Con A administration induces expression of TLR2, TLR3, TLR4, and TLR9 in macrophages in vivo. In addition, Con A-induced TNF-α expression in macrophages is downregulated in MyD88-deficient mice. It is well known that damage-associated molecular pattern molecules (DAMPs) are released from cells undergoing necrotic cell death, and then recognized by TLRs and other pattern recognition receptors. Liver macrophages can sense DAMPs, thereby producing inflammatory cytokines. From these reports and our results, we speculate that Con A administration induces the release of DAMPs from hepatocytes, activating the TLR-MyD88-IRF5 signaling cascade in liver macrophages.

Further modifications to the biodegradable LNPs may allow them to be more efficiently incorporated into macrophages and other immune cell populations. LNPs larger than 200 nm in diameter are more

Figure 3. Role of ApoE in biodegradable LNP uptake into macrophages

(A) Flow cytometry analysis of AF488-labeled siRNA distribution following intravenous injection of LNPs in Apoe–/– mice. F4/80+CD11b+ macrophages and B cells in the liver were analyzed 3 h after intravenous injection of LNP-encapsulated AF488-labeled siRNA at a siRNA dose of 0.8 mg/kg. MFI of AF488 in each cell population is shown in the boxplots. Values from two independent experiments are shown. (B) Irf5 mRNA expression analysis following intravenous injection of siirf5-LNPs in Apoe–/– mice. Mice were injected with siirf5-LNPs or siCtrl-LNPs at a siRNA dose of 5.0 mg/kg. Twenty-four hours after LNP administration, Irf5 mRNA expression in liver F4/80+CD11b+ Mφs and B cells were analyzed by qRT-PCR. Data from three independent experiments are shown. *p < 0.05 (Student’s t test). WT, wild-type.
readily taken up by peritoneal macrophages compared to smaller LNPs.\(^{39}\) As the size of our LNPs is approximately 70–80 nm (see Figure S1), increasing their size might further improve the efficacy. In addition, mannosylated LNPs are effectively incorporated into mannose receptor-expressing macrophages such as tumor-associated macrophages.\(^{39}\) LNPs coated with antibodies targeting DEC205 and CD4 were capable of efficiently delivering siRNA to a dendritic cell subpopulation and CD4\(^+\) T cells in mice, respectively.\(^{60,61}\) Such modifications could improve the efficiency and versatility of our LNPs and extend their potential applications for clinical use in the future.

**MATERIALS AND METHODS**

**Mice**

Male and female wild-type, Irf5\(^−/−\), \(^{35}\) and Apoe\(^−/−\) mice 8–12 weeks old in a C57BL/6 background were used. All animal experiments were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan), and all protocols were approved by the Institutional Review Boards of Yokohama City University (protocols F-A-17-018 and F-A-20-043).

**siRNA synthesis**

siIrf5 and siRNA targeting luciferase (siCtrl) have been described in previous reports.\(^{16,18}\) AF488-labeled siRNA (sense, 5’-AcAuGAAG cAGcACGAcuUdTsdT-3’, antisense, 5’-AAUGUuCUCUCUUCAGUdTsdT-aminoC6 linker-AF488-3’), siIrf5 (sense, 5’-cuGeAG GSSuSSccuGAdTsdT-3’, antisense, 5’-uCAggGuaUUCCUCUCGc AGdTsdT-3’), and siCtrl (sense, 5’-cuAcAcGcuGAGuAcuecGA dTsdT-3’, antisense, 5’-UCGAAAGuACucGcuAAdTsdT-3’) were synthesized by GeneDesign (Japan). Lowercase letters denote 2’-O-methyl (2’-OMe)-modified residues.

**Formulation of biodegradable LNPs**

Biodegradable LNPs were prepared as previously described.\(^{15}\) Briefly, siRNA was dissolved in 10 mM sodium citrate (0.45 mg/mL). Biodegradable ionized lipid L120,\(^{31}\) 1,2-distearyl-sn-glycerol-3-phosphocholine (DSPC), cholesterol, and 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol (PEG-DMG) at a molar ratio of 60:8.5:30:1.5 were then dissolved in ethanol. The total lipid concentration was 40 mM. Using two syringe pumps, siRNA solutions and lipid solutions were mixed at a flow rate of 3 and 1 mL/min, respectively. The mixing ratio of siRNA and lipids was 0.06 w/w. After overnight dialysis with PBS (pH 7.4) using a 100-kDa dialysis tube, the solution was sterilized using a 0.22-μm membrane filter. The physical properties of our LNPs are shown in Figure S1.

**Cell isolation and flow cytometry**

Hepatic immune cells were prepared as previously described.\(^{62}\) Briefly, after perfusion with 0.5 mg/mL collagenase type 4 (Worthington Biochemical) via the portal vein, liver tissue was minced and homogenized using a 70-μm cell strainer (Corning Life Sciences). Liver cells were resuspended in 45% Percoll solution and centrifuged at 50 × g for 10 min to enrich liver-infiltrating immune cells. Spleen cells were obtained with Liberase and DNase I (Roche) treatment.\(^{63}\) Bone marrow cells were harvested by flushing femurs and tibias.\(^{63}\) Splenocytes and bone marrow cells were treated with red blood cell lysis solution. Peritoneal cells were obtained by washing the peritoneal cavity with RPMI 1640 media containing 10% fetal bovine serum (FBS). These cells were stained with fluorochrome-labeled antibodies. Flow cytometry was performed using FACSCanto II and FACSaria II (BD Biosciences), and data were analyzed using FlowJo software (FlowJo).

**Markers and antibodies used for flow cytometric analysis**

Markers used for flow cytometry in this study were as follows: hematopoietic stem cells, lineage markers (Lin; CD5, B220, CD11b, Ly6G/C, 7-4, Ter-119) Sca-1\(^+/−\) CD117\(^−/−\) IL-7R\(^+/−\) CD150\(^+/−\) CD34\(^+/−\)), lymphoid-primed multipotent progenitors, Lin Sca-1\(^+/−\) CD117\(^−/−\) IL-7R\(^+/−\) CD150\(^+/−\) CD34\(^+/−\) Flt3\(^+\)), common myeloid progenitors, Lin CD11b\(^+\) IL-7R\(^+/−\) CD115\(^+/−\) CD34\(^+/−\) CD16/32\(^−\)), granulocyte monocyte progenitors,
Lin^CD117^hi(IL-7R^CD115^CD34^CD16/32^hi), megakaryocyte erythrocyte progenitors, Lin^CD117^hi(IL-7R^CD115^CD34^CD16/32^), monocyte dendritic cell progenitors, Lin^CD117^hi(IL-7R^CD115^Flt3^); common dendritic cell progenitors, Lin^CD117^himid/lo(IL-7R^CD115^Flt3^); committed classical dendritic cell precursors, Lin^major histocompatibility complex (MHC) class II^CD11c^Flt3^; common monocyte progenitors, CD3^CD19^Ly6G^NK1.1^CD115^CD117^Flt3^Ly6C^CD11b^; classical dendritic cells, MHC class II^CD11c^hi; plasmacytoid dendritic cells, B220^PDCA-1^CD11b^CD11c^hi, monocytes, CD11b^CD115^Ly6G^; neutrophils, CD11b^Ly6G^; eosinophils, Ly6G^F4/80^CD11b^Siglec F^; basophils, CD11^CD49b^CD200R3^; B cells, CD3^B220^CD19^CD11b^CD11c^; T cells, CD3^TCR-B^CD11c^; and NK cells, NK1.1^CD49b^*. Macrophages are identified by F4/80 and CD11b staining. Antibodies used in this study and their clone names are as follows: anti-B220 (RA3-6B2), CD115 (AF588), CD117 (2B8), CD11b (M1/70), CD11c (N418), CD150 (TC15-12F12.2), CD16/32 (93), CD200R3 (Ba160), CD3e (145-2C11), CD4 (GK1.5), CD49b (DX5), CD8 (53-6.7), CD19 (6D5), F4/80 (BM8), Fli3 (A2F10), IL-7R (A7R34), Ly6C (HK1.4), Ly6G (1A8), MHC class II (AF6-120.1), NK1.1 (PK136), PDCA-1 (927), Sca-1 (E13-161.7), TCR-β (H57-597) (BioLegend), CD34 (RAM34) (eBioscience), and Siglec F (E50-2440) (BD Biosciences).

**Intracellular IRF5 staining**

Liver cells were stained with antibodies for surface markers as described above. The cells were then fixed in 4% paraformaldehyde and permeabilized with 90% methanol. Rabbit anti-IRF5 (ab21689; Abcam) and R-phycocerythrin-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch) were used as primary and secondary antibodies, respectively.

**qRT-PCR**

Total mRNA was extracted using an RNasy mini kit (QIAGEN) and reverse transcribed using the PrimeScript RT reagent kit (Takara) according to the manufacturer’s instructions. The following primers were used in qRT-PCR analyses: Irf1 (sense, 5'-CCC AGG GCT GAT CTG GAT CAA TAA A-3'; antisense, 5'-ACA GAC AGG CAT CCT TGT TGA TGT G-3'), Irf2 (sense, 5'-TTT AAC TGA CCG GCT TTC ATT TCC A-3'; antisense, 5'-CAG CCG CAT GGT ACC CTC TCA A-3'), Irf3 (sense, 5'-TGG CAG GAC TAC TAC TGT TTG G-3'; antisense, 5'-GGT TCC ATG CTC TAG CCA GGG G-3'), Irf4 (sense, 5'-AAG GCC CAT CCT GTG AAA ATG GTT G-3'; antisense, 5'-ATT CTG GTC GGA ATG GCA AGG GAA AAG AAA C-3'), Irf5 (sense, 5'-CAG TGG GTC AAC GGG GAA AAG AAA C-3'; antisense, 5'-CGG CTG GCC GCT AAC ATG GGT G-3'), Irf6 (sense, 5'-CAC GGA CTG AGG CCA GAT CAT-3'; antisense, 5'-TGG AAG CGT TTG GAA TCT CTG TGT A-3'), Irf7 (sense, 5'-ACA GCA CAG GCC GTC TTA TC-3'; antisense, 5'-GAG CCC ACC ATG ATT TCC TCT TG-3'), Irf8 (sense, 5'-GCA GGA GTG ACC GAA AAC AC-3'; antisense, 5'-GGA TAC GGA ACA ATG GTC TT-3'), Gapdh (sense, 5'-GGT TTC CTA CCC CCA ATG T-3'; antisense, 5'-TGG CAT ACT TGG CAG CTT TC-3'). Data were analyzed using the ΔΔCt method and normalized against Gapdh expression levels.

**Induction of Con A-induced liver injury**

Con A (Vector Laboratories) was dissolved in PBS and administered to mice through intravenous injection (20 mg/kg body weight). Serum AST and ALT levels were quantified 12 h after Con A injection, plasma AST and ALT levels were measured. (C, left) H&E staining of the liver sections 12 h after Con A injection was performed. Scale bars represent 200 μm. (C, right) Percentages of necrotic area were calculated using ImageJ software. (D) TNF-α and IL-6 levels: anti-B220 (RA3-6B2), CD115 (AF588), CD117 (2B8), CD11b (M1/70), CD11c (N418), CD150 (TC15-12F12.2), CD16/32 (93), CD200R3 (Ba160), CD3e (145-2C11), CD4 (GK1.5), CD49b (DX5), and normalized against Gapdh (sense, 5'-ACA GCA CAG GCC GTC TTA TC-3'; antisense, 5'-GGA TAC GGA ACA ATG GTC TT-3'). Data were analyzed using the ΔΔCt method and normalized against Gapdh expression levels.

**Quantification of AST and ALT levels**

Serum AST and ALT levels were quantified by a 7180 clinical analyzer (Hitachi) at Oriental Yeast.

**Histology**

Livers were harvested 12 h after Con A injection. Tissues were fixed in 10% buffered formalin. Formalin-fixed and paraffin-embedded liver sections were cut and stained with hematoxylin and eosin at the Sapporo General Pathology Laboratory. Necrotic areas were measured in each section using ImageJ software (https://imagej.nih.gov/ij/).
Enzyme-linked immunosorbent assay (ELISA)

After administration of Con A, blood was collected through tail veins at specified time points. Serum was analyzed using the mouse TNF-α ELISA MAX standard kit and mouse IL-6 ELISA MAX standard kit (BioLegend).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.08.023.

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DECLARATION OF INTERESTS

Y.S., H.I., and K.T. are employees of Eisai Co., Ltd. T. Tamura received a joint research fund from Eisai Co., Ltd. The remaining authors declare no competing interests.

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