Systemic PEGylated TRAIL Treatment Ameliorates Liver Cirrhosis in Rats by Eliminating Activated Hepatic Stellate Cells

Yumin Oh,[1,2]* Ogyi Park,[1–3]* Magdalena Swierczewska,[1,2]* James P. Hamilton,[4] Jong-Sung Park,[1,2] Tae Hyung Kim,[1,2]
Sung-Mook Lim,[5] Hana Eom,[5] Dong Gyu Jo,[5] Choong-Eun Lee,[6] Raouf Kechrid,[3] Panagiotis Mastorakos,[2] Clark Zhang,[2]
Sei Kwang Hahn,[7] Ok-Cheol Jeon,[8] Youngro Byun,[8] Kwangmeyung Kim,[9] Justin Hanes,[2] Kang Choon Lee,[5]
Martin G. Pomper,[1] Bin Gao,[3] and Seulki Lee[1,2,10]

Liver fibrosis is a common outcome of chronic liver disease that leads to liver cirrhosis and hepatocellular carcinoma. No US Food and Drug Administration–approved targeted antifibrotic therapy exists. Activated hepatic stellate cells (aHSCs) are the major cell types responsible for liver fibrosis; therefore, eradication of aHSCs, while preserving quiescent HSCs and other normal cells, is a logical strategy to stop and/or reverse liver fibrogenesis/fibrosis. However, there are no effective approaches to specifically deplete aHSCs during fibrosis without systemic toxicity. aHSCs are associated with elevated expression of death receptors and become sensitive to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)-induced cell death. Treatment with recombinant TRAIL could be a potential strategy to ameliorate liver fibrosis; however, the therapeutic application of recombinant TRAIL is halted due to its very short half-life. To overcome this problem, we previously generated PEGylated TRAIL (TRAIL$_{PEG}$) that has a much longer half-life in rodents than native-type TRAIL. In this study, we demonstrate that intravenous TRAIL$_{PEG}$ has a markedly extended half-life over native-type TRAIL in nonhuman primates and has no toxicity in primary human hepatocytes. Intravenous injection of TRAIL$_{PEG}$ directly induces apoptosis of aHSCs *in vivo* and ameliorates carbon tetrachloride-induced fibrosis/cirrhosis in rats by simultaneously down-regulating multiple key fibrotic markers that are associated with aHSCs. *Conclusion*: TRAIL-based therapies could serve as new therapeutics for liver fibrosis/cirrhosis and possibly other fibrotic diseases. (HEPATOLOGY 2016;64:209-223)

SEE EDITORIAL ON PAGE 29

The mechanisms that underlie the pathogenesis of liver fibrosis have been studied extensively, yet no medications have emerged as effective antifibrotic agents. Chronic liver injury from liver diseases such as viral hepatitis, alcoholic hepatitis, nonalcoholic steatohepatitis, biliary diseases, metabolic disorders, or autoimmune conditions stimulates the accumulation of excessive extracellular matrix (ECM) that results in liver fibrosis. Progressive liver fibrosis leads to cirrhosis and remodeling of the hepatic vascular architecture that can

result in liver failure, cancer, and premature death.[1-4] Hepatic stellate cells (HSCs) are the major cell type that produce excessive ECM, leading to liver fibrosis. Quiescent HSCs (qHSCs) are initially activated by several factors such as damaged hepatocytes, apoptotic bodies, cytokines, and chemokines produced by resident hepatic macrophages (Kupffer cells) and infiltrating inflammatory cells during liver injury. Activated hepatic stellate cells (aHSCs) express platelet-derived growth factor (PDGF) and PDGF receptors (PDGF-Rs). PDGF induces HSC proliferation, resulting in increased production of profibrogenic cytokines such as transforming growth factor-β (TGF-β), which further activate HSCs to up-regulate α smooth muscle actin (α-SMA) expression and stimulate ECM secretion. aHSCs and Kupffer cells also express tissue inhibitors of metalloproteinases (TIMPs) which inhibit matrix-degrading metalloproteinase activity and promote HSC survival, altering the balance between ECM secretion and degradation. Based on their role in the fibrotic cascade, aHSCs are a major target for antifibrotic therapy.[5,6] Selectively eradicating aHSCs but not qHSCs or other liver cells is anticipated to induce strong antifibrotic effects, because the originator cells of fibrogenesis are depleted and key fibrogenic components are simultaneously inhibited. However, there is a lack of robust methods by which aHSCs may be inactivated or eradicated. Moreover, aHSCs are known to be resistant to apoptotic stimuli, including Fas ligand (FasL), tumor necrosis factor α (TNF-α), and DNA intercalating agents such as anticancer drugs and oxidative stress mediators.[7]

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a type 2 transmembrane protein in the TNF-α superfamily due to sequence homology with TNF and FasL.[8,9] TRAIL can be proteolytically cleaved from the cell surface and released in soluble form. Soluble TRAIL is intrinsically a homotrimer and subsequently trimerizes TRAIL receptors after binding. Five TRAIL receptors have been identified in humans, but only two TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors initiate apoptosis similar to Fas/FasL and TNF-R/TNF signaling pathways. TRAIL receptor binding stimulates formation of death-inducing signaling complex (DISC) with the recruited adaptor protein, Fas-associated protein with death domain (FADD). FADD recruits procaspases 8 and 10, and DISC allows auto-activation of these caspases. Downstream of this signaling is the proteolytic cleavage and activation of caspases 3, 6, and 7, resulting in apoptosis. Another pathway of apoptosis is the induction of mitochondrial dysfunction and membrane

ARTICLE INFORMATION:

From the [1]The Russell H. Morgan Department of Radiology and Radiological Science, Johns Hopkins University School of Medicine, Baltimore, MD; [2]The Center for Nanomedicine at the Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD; [3]Laboratory of Liver Diseases, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD; [4]Division of Gastroenterology and Hepatology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD; [5]College of Pharmacy; [6]Department of Biological Science, Sungkyunkwan University, Suwon, Korea; [7]Department of Materials Science and Engineering, Pohang University of Science and Technology, Pohang, Korea; [8]College of Pharmacy, Seoul National University, Seoul, Korea; [9]Center for Theragnosis, Korea Institute of Science and Technology, Seoul, Korea; [10]Therapy Pharmaceuticals, Inc., Baltimore, MD.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Bin Gao, M.D., Ph.D.
Laboratory of Liver Diseases
NIAAA/NIH, 5625 Fishers Lane
Bethesda, MD 20892
E-mail: bgao@mail.nih.gov
Fax: 301-480-0257
or

Seulki Lee, Ph.D.
The Russell H. Morgan Department of Radiology and Radiological Science
The Center for Nanomedicine at the Wilmer Eye Institute
Johns Hopkins University, School of Medicine
Baltimore, MD 21231
E-mail: seulki@jhmi.edu
Fax: 410-614-6509

permeabilization causing release of cytochrome c that activates caspase 9 and finally cleavage of caspases 3 and 7, resulting in apoptosis. TRAIL can also bind to its decoy receptors, TRAIL-R3/DcR1 and TRAIL-R4/DcR2, but these receptors lack a functional death domain and are unable to induce apoptosis. Dulanermin, or recombinant human TRAIL, has been investigated as an anticancer therapy; however, its clinical efficacy has been disappointing,[10,11] probably due to TRAIL resistance in primary cancer cells and the short half-life of the protein (<5 minutes in rodents and <30 minutes in humans).[12,13]

The aHSC cell line LX2 up-regulates DR4 and DR5 and becomes sensitive to TRAIL-induced cell death.[14] Despite promising *in vitro* studies, the role of TRAIL signaling in liver fibrogenesis has not been investigated fully. Furthermore, an effective molecule that can selectively induce apoptosis in aHSCs with limited hepatotoxicity has not been developed, so the translation of antifibrotic therapies from "bench to bedside" has been limited. We sought to determine whether such a strategy has therapeutic potential in liver fibrosis and cirrhosis. We verified whether TRAIL is a suitable target for antifibrotic therapy by comparing TRAIL receptor expression levels in activated primary human HSCs and in liver tissue samples from healthy patients and in patients with liver fibrosis/cirrhosis. To address the poor clinical potency of recombinant TRAIL in oncologic clinical studies, we used a long-acting TRAIL consisting of a PEGylated human trimeric isoleucine-zipper fused TRAIL (TRAIL$_{PEG}$). The antifibrotic potency of long-acting TRAIL was investigated in carbon tetrachloride (CCl$_4$)-induced fibrosis rat models at various stages of injury. We explore the role of systemic TRAIL$_{PEG}$ in liver fibrogenesis *in vivo* and mechanisms of TRAIL sensitization in primary human HSCs. The results warrant further investigation into stable TRAIL-based materials as antifibrotic therapeutic strategies.

Materials and Methods

HUMAN LIVER SAMPLES

The Liver Tissue Procurement Distribution System (LTPDS; Division of Pediatric Gastroenterology and Nutrition, University of Minnesota, Minneapolis, MN) provided frozen alcoholic cirrhotic liver samples and paraffin-embedded liver samples from patients with hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease (ALD), or ALD/HCV with end-stage cirrhosis who underwent liver transplantation. Liver disease diagnoses were made by the LTPDS and were based on a history of alcohol drinking, infected viral markers, and liver histology. Liver pathology of these specimens showed bridging fibrosis and cirrhosis. The LTPDS also provided paraffin-embedded normal healthy liver specimens obtained from human donor livers not used for transplantation. The protocol for using the liver samples was approved by the LTPDS of the University of Minnesota and the US National Institutes of Health. Frozen normal human liver tissues were purchased from Triangle Research Labs (Durham, NC) for analysis of protein expression by western blotting as controls.

HUMAN PRIMARY HEPATOCYTE CULTURE AND TRAIL$_{PEG}$ TREATMENT

Cryopreserved human primary hepatocytes, human hepatocyte plating medium, and thawing medium were obtained from Triangle Research Labs. According to the manufacturer's instructions, cryopreserved hepatocytes were thawed in thawing medium and cultured in human hepatocyte plating medium in a 6-well plate of collagen type I Biocoat (BD Biosciences, San Jose, CA). Cells were cultured overnight and then treated with TRAIL$_{PEG}$ or recombinant human His-iLZ-TRAIL for 3 hours. After cells were harvested, the expression of TRAIL receptors (DR4/DR5) and apoptosis markers were determined by way of western blot analysis. Cell viability was analyzed using MTT assays.

LIVER FIBROSIS AND CIRRHOSIS INDUCED BY CCl$_4$ IN RATS

Animal studies were undertaken according to an approved protocol reviewed by the Johns Hopkins Animal Care and Use Committee. Male Sprague-Dawley rats (age, 5-6 weeks; body weight, 120-150 g) were purchased from Charles River (Germantown, MD). Rats were divided into four groups: (1) olive oil and phosphate-buffered saline (PBS) treated groups, (2) olive oil and TRAIL$_{PEG}$, (3) CCl$_4$ and PBS and (4) CCl$_4$ and TRAIL$_{PEG}$. For fibrotic rats, rats were administered with 2 mL/kg of CCl$_4$ (Sigma-Aldrich, 20% CCl$_4$ in olive oil) three times per week through intraperitoneal injection or olive oil as control groups for a total of 4 weeks. At day 29, rats were treated with 4 mg/kg of TRAIL$_{PEG}$ through intravenous injection every day for 10 days or were treated with the same

amount of PBS for control groups. Rats were anesthetized at day 40, and blood and liver tissues were collected for analysis. To induce liver cirrhosis, rats were divided into four groups just as the fibrosis groups and administered with CCl$_4$ (20% CCl$_4$ in olive oil, 2 mL/kg) three times per week via intraperitoneal injection or olive oil as control groups for a total of 7 weeks. Starting on day 50, rats were treated with 4 mg/kg of TRAIL$_{PEG}$ through intravenous injection every day for 14 days or were treated with the same amount of PBS for control groups. Rats were anesthetized at day 65, and blood and liver tissues were collected for analysis.

STATISTICAL ANALYSIS

All data were analyzed using GraphPad Prism 6 software. Differences between two means were assessed using a paired or unpaired t test. Differences among multiple means were assessed, as indicated, by one-way analysis of variance followed by Tukey's *post hoc* test or a Student t test as appropriate. Error bars represent standard deviation (SD) or standard error of the mean (SEM), as indicated. Probabilities of $P < 0.05$ or as indicated were considered statistically significant.

OTHER METHODS

Additional methods are described in the Supporting Information.

Results

TRAIL$_{PEG}$ HAS AN EXTENDED HALF-LIFE *IN VIVO* WITHOUT TOXICITY IN PRIMARY HUMAN HEPATOCYTES

The extremely short half-life and low *in vivo* potency of recombinant human TRAIL make it difficult to provide continuous and potent TRAIL-induced cell apoptosis. In addition, many TRAIL-based therapies have been unstable in solution and can aggregate at high concentrations, leading to toxicity and dose limitations in clinical studies. To overcome these disadvantages, we developed TRAIL$_{PEG}$ by stabilizing a potent homotrimer TRAIL comprised of isoleucine-zipper amino acid motifs at the N-terminus that favor trimer formation (His-iLZ-TRAIL) with a 5-kDa poly(ethylene glycol) (PEG).[15,16] PEGylation is a highly efficient commercial strategy to extend the half-life of protein drugs as well as reduce protein

aggregation.[17,18] We demonstrated that TRAIL$_{PEG}$ has an improved pharmacodynamic profile in a tumor xenograft model and an improved pharmacokinetic profile in rats compared with recombinant TRAIL.[15] TRAIL$_{PEG}$ demonstrated a substantially extended half-life (8.6 hours) in cynomolgus monkeys after intravenous injection compared with His-iLZ-TRAIL (0.9 hours) (Fig. 1A and Supporting Table 1). Native-type TRAIL is nontoxic to primary hepatocytes; because hepatocytes, like many nontransformed cells, are resistant to TRAIL-induced apoptosis despite expressing TRAIL receptors.[19] However, some TRAIL variants, particularly His-tagged or Flag-tagged TRAIL, are prone to uncontrolled aggregation and induce pronounced apoptosis in hepatocytes.[20,21] To investigate potential liver toxicity, primary human hepatocytes were treated *in vitro* with varying concentrations of TRAIL$_{PEG}$, and apoptotic signaling and cell death was compared against its non-PEGylated analog. His-iLZ-TRAIL induced apoptosis in human hepatocytes *in vitro* at concentrations higher than 100 ng/mL as evidenced by increased cleaved PARP-1 and cleaved caspases (Fig. 1B) as well as by quantified cell death (Fig. 1C). In contrast, TRAIL$_{PEG}$ did not show toxicity at concentrations up to 5000 ng/mL. Steatotic hepatocytes, which are known to be more sensitive to TRAIL-mediated cytotoxicity,[22] undergo apoptosis when treated with high concentrations of His-iLZ-TRAIL but demonstrate no cell death with TRAIL$_{PEG}$ at the same TRAIL concentration (Supporting Fig. 1). Despite having a lower toxicity profile, TRAIL$_{PEG}$ maintained equal cancer killing efficiency when compared with His-iLZ-TRAIL with significantly improved solubility (reduced aggregation) at high concentrations in neutral pH, as we demonstrated previously.[15]

TRAIL RECEPTOR EXPRESSION LEVELS ARE UP-REGULATED IN HSCS IN HUMAN CIRRHOTIC LIVERS

To validate the clinical relevance of our strategy, we measured TRAIL receptor expression levels in aHSCs/myofibroblasts from liver sections of patients with liver cirrhosis. Immunohistochemical analysis and immunofluorescence double staining revealed that DR4 and DR5 expression was elevated in human cirrhotic liver tissues associated with HBV, HCV infection, or chronic alcohol consumption (Supporting Fig. 2) and colocalized with α-SMA$^+$ aHSCs (Fig. 1D).
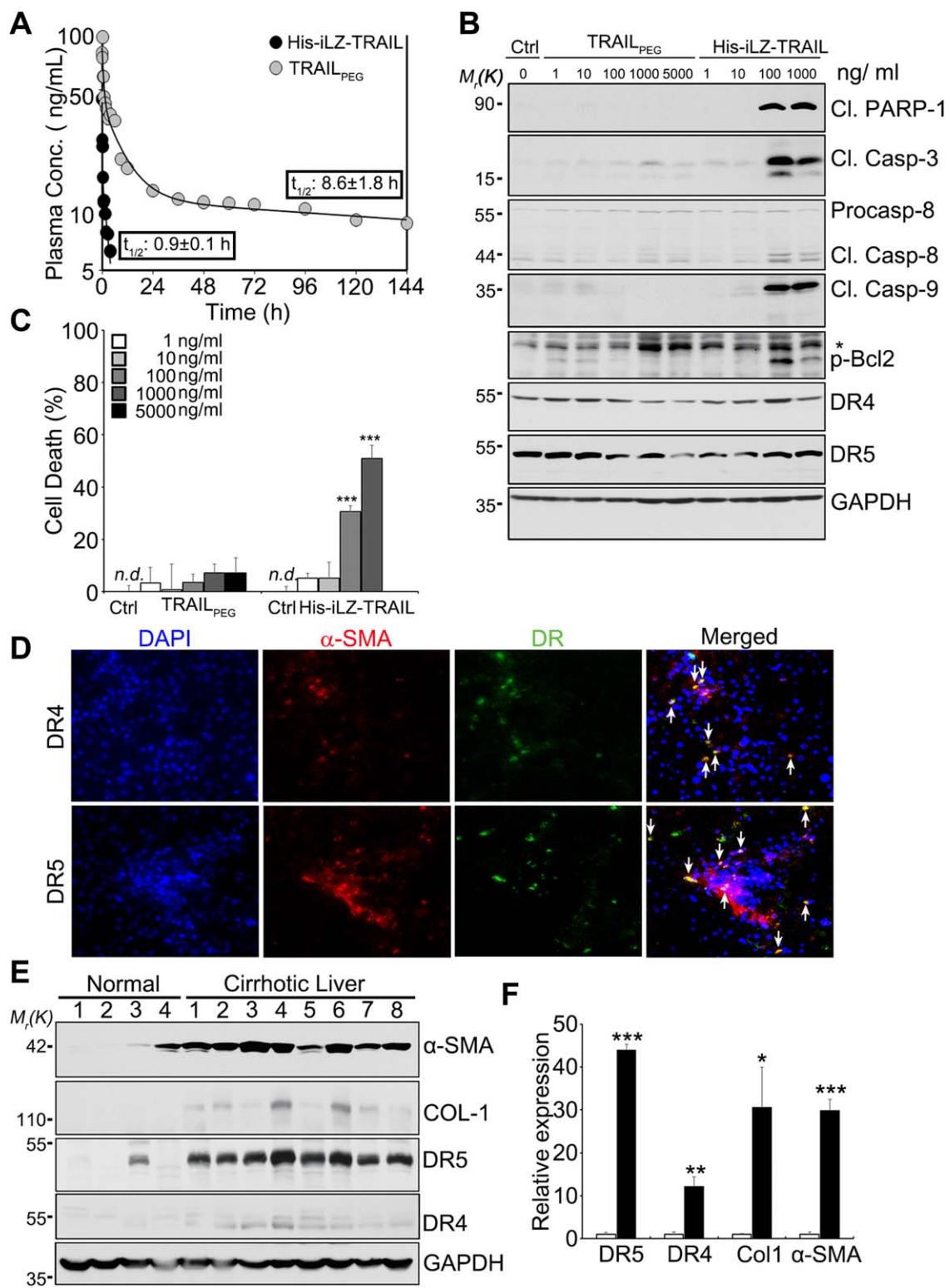
FIG. 1. Intravenously injected TRAIL$_{PEG}$ shows extended half-life in nonhuman primates and no toxicity in primary human hepatocytes. TRAIL receptors, DR5 and DR4, are up-regulated in human cirrhotic livers and colocalized in aHSCs. (A) Pharmacokinetic profiles of His-iLZ-TRAIL and TRAIL$_{PEG}$ (12.5 μg/kg) after intravenous injection in cynomolgus monkeys (n = 2 per group) labeled with quantified half-lives. (B, C) Safety of TRAIL$_{PEG}$ in primary human hepatocytes. The control (Ctrl) group was untreated. (B) Western blot analysis of hepatocytes treated with various concentrations of TRAIL$_{PEG}$ or His-iLZ-TRAIL for 3 hours. (C) Quantified cell death analyzed by way of MTT assay after treatment of primary hepatocytes with TRAIL$_{PEG}$ or His-iLZ-TRAIL. Data are expressed as the mean ± SD. ***$P < 0.001$ versus nontreated groups (Ctrl). n.d., nondetectable. (D) Double immunofluorescence micrographs of cirrhotic liver sections stained for nuclei (DAPI, blue) with DR4 or DR5 (green) and α-SMA (red). Arrows indicate colocalized DR4 or DR5 with α-SMA–positive cells, aHSCs (original magnification ×200). (E) Western blot analyses of human normal (four human samples, lanes 1-4) and alcoholic cirrhotic liver tissues (eight patient samples, lanes 1-8). (F) Densitometry analysis of western blots from panel E shown as relative protein expression normalized to healthy liver tissue. Data are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus nontreated groups (Ctrl).

Western blot analyses of liver tissues from patients with alcoholic cirrhosis showed strong up-regulation of DR5 and moderate up-regulation of DR4 compared with healthy livers (Fig. 1E,F). These data validate DRs as a clinical target for aHSCs and imply that TRAIL-based molecules can target aHSCs. By verifying DR expression on aHSCs and developing a stable and safe TRAIL receptor agonist with a prolonged pharmacokinetic profile in nonhuman primates, we were motivated to explore TRAIL$_{PEG}$ and its ability to eradicate aHSCs in preclinical models of liver fibrosis and cirrhosis.

INTRAVENOUSLY INJECTED TRAIL$_{PEG}$ AMELIORATES CCL$_4$-INDUCED LIVER FIBROSIS IN RATS

To investigate the effect of TRAIL$_{PEG}$ on fibrosis *in vivo*, liver fibrosis was induced by thrice-weekly administration of CCl$_4$ in rats,[23] as shown in the treatment schedule in Fig. 2A. Two control groups received olive oil alone. After 4 weeks of CCl$_4$ exposure, rats with fibrosis received TRAIL$_{PEG}$ (4 mg/kg, protein-based, at day 29) or PBS daily for a total of 10 days while continuing to receive CCl$_4$. Olive oil served as the vehicle control for CCl$_4$ exposure in rats, and PBS served as the vehicle control for TRAIL$_{PEG}$ treatment. In the rats with CCl$_4$-induced liver fibrosis, liver weight-body weight ratio was significantly lower in TRAIL$_{PEG}$-treated rats compared with the PBS-treated group (Fig. 2B). Alkaline phosphatase and total bilirubin (Fig. 2C) were significantly lower in sera from TRAIL$_{PEG}$-treated fibrotic rats than PBS-treated fibrotic rats. Alanine aminotransferase and aspartate aminotransferase levels were not significantly different between the TRAIL$_{PEG}$- and PBS-treated groups, likely due to continuous CCl$_4$-induced liver damage during the study (Supporting Fig. 3). Immunohistochemistry and computerized image analyses clearly showed markedly reduced positive areas of fibrosis detected by α-SMA and collagen deposition staining (sirius red) in liver specimens from TRAIL$_{PEG}$-treated rats compared with the PBS-treated group (Fig. 3D,E). In addition, hydroxyproline levels, a quantification of collagen deposition in liver tissue, were lower in the TRAIL$_{PEG}$-treated disease model over the untreated (PBS) study group (Fig. 3F). Fibrotic and TRAIL signaling markers in liver tissues during CCl$_4$ and TRAIL$_{PEG}$ treatments were analyzed at messenger RNA (mRNA) and protein levels. Quantitative real-time polymerase chain reaction

(qRT-PCR) of mRNA obtained from TRAIL$_{PEG}$-treated liver tissues revealed an obvious reduction of multiple, highly up-regulated genes associated with the transition of qHSCs to the aHSC/myofibroblast phenotype, including Dr5 (TRAIL-R), Acta-2 (α-SMA), Col1a2 (collagen I), Col3a1 (collagen III), Tgf-β1 (transforming growth factor β1), Bmp-7 (bone morphogenetic protein-7), Pdgf-r (platelet-derived growth factor receptors [PDGF-R]), Mmp-2 and -13 (matrix metalloproteinases 2 and 13), and Timp-1 and -3 (tissue inhibitors of metalloproteinase 1 and 3) (Fig. 3A). Western blot analyses confirmed a significant decrease in expression levels of these proteins in the TRAIL$_{PEG}$-treated group (Fig. 3B and Supporting Fig. 4). All the tested markers were statistically reduced by at least 50% at mRNA and protein levels. To verify selective TRAIL-induced apoptosis in aHSCs, we double-stained the liver sections from control (olive oil) or CCl$_4$-induced fibrotic rats treated with PBS or TRAIL$_{PEG}$ for α-SMA$^+$ aHSCs and apoptosis using terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL). In control (i.e., olive oil-treated) healthy livers, no strong α-SMA or TUNEL staining was observed. In TRAIL$_{PEG}$-treated fibrotic livers, TUNEL staining colocalized with α-SMA, validating that apoptosis occurred in α-SMA$^+$ aHSCs (Fig. 3C and Supporting Fig. 5). Importantly, continuous systemic administration of TRAIL$_{PEG}$ in oil alone to normal rats did not induce any noticeable toxicity, particularly in the liver. Animal groups treated with olive oil/PBS and olive oil/TRAIL$_{PEG}$ demonstrated the same levels of alanine aminotransferase and aspartate aminotransferase (Supporting Fig. 3).

INTRAVENOUSLY INJECTED TRAIL$_{PEG}$ AMELIORATES CCL$_4$-INDUCED LIVER CIRRHOSIS IN RATS

After demonstrating clear antifibrotic activity of TRAIL$_{PEG}$ in liver fibrosis, we hypothesized that it can also reverse the fibrotic process in cirrhotic livers. In cirrhotic rats after long-term CCl$_4$-treatment,[23] TRAIL$_{PEG}$ (4 mg/kg, protein-based, at day 50) was injected daily for 14 days along with continued CCl$_4$-treatment, and samples were collected and examined at day 65 of CCl$_4$ treatment (Fig. 4A). Just as in the fibrosis study, olive oil served as the vehicle control for CCl$_4$ exposure in rats, and PBS served as the vehicle control for TRAIL$_{PEG}$ treatment. As illustrated in
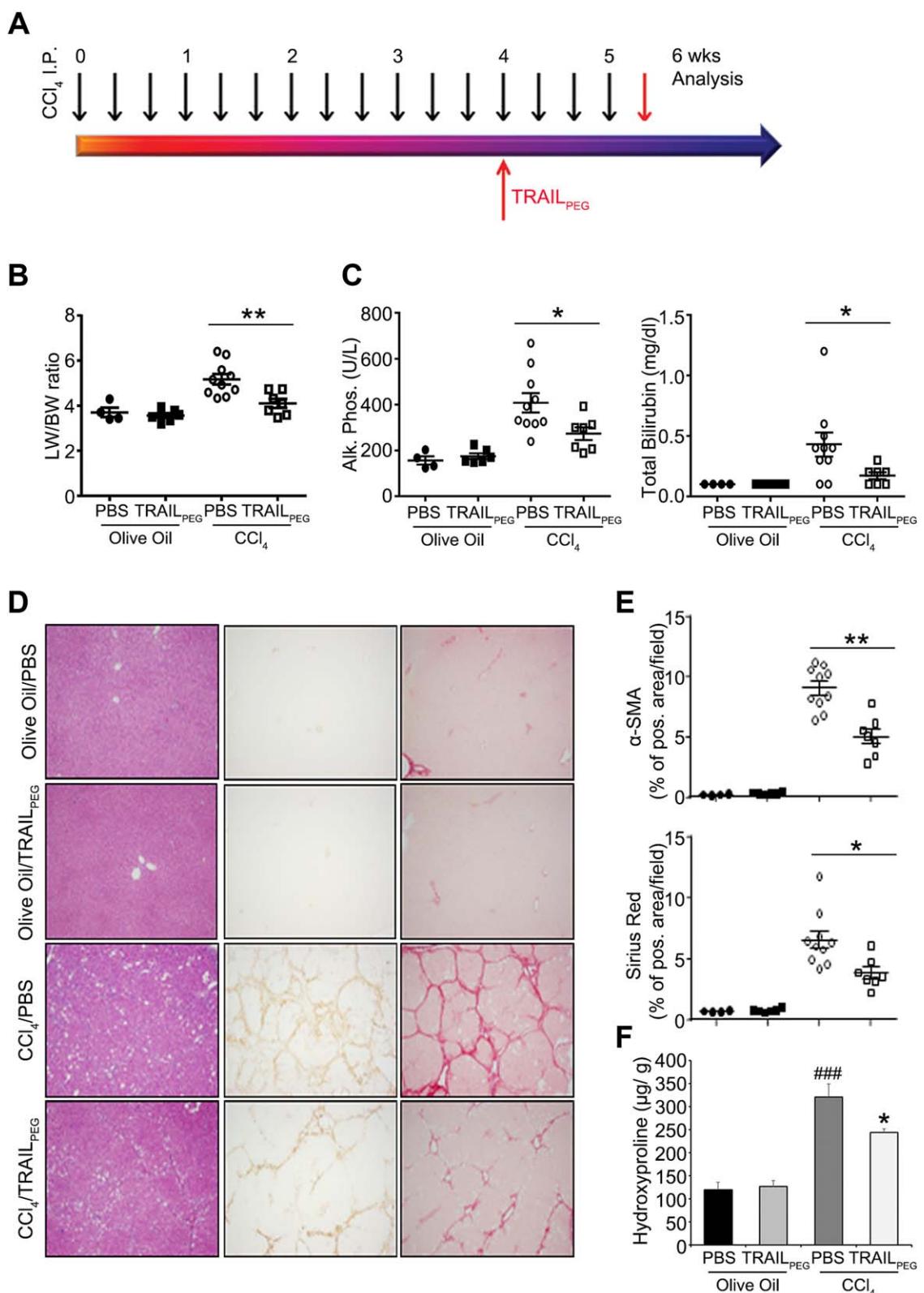
FIG. 2. Intravenously injected TRAIL$_{PEG}$ ameliorates CCl$_4$-induced liver fibrosis in rats. (A) Intravenous TRAIL$_{PEG}$ (4 mg/kg) was administered daily in control and in fibrotic livers induced by CCl$_4$ (three times per week) in rats. TRAIL$_{PEG}$ or PBS treatment was initiated at day 29 in control (olive oil–treated) and CCl$_4$ rats. Livers and blood samples were obtained at day 40. (B) Liver weight/body weight (LW/BW) analyses. (C) Serum levels of alkaline phosphatase and total bilirubin. (D) Representative photomicrographs of liver sections from control (olive oil–treated) or chronic CCl$_4$ rats with or without TRAIL$_{PEG}$ stained with hematoxylin and eosin and immunohistochemistry of activated HSC marker (α-SMA) and collagen deposition (sirius red) (original magnification ×40). (E) Digital image quantification of α-SMA and sirius red (collagen) staining. (F) Quantification of collagen by hydroxyproline analysis in total livers. Data are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus CCl$_4$ + PBS groups. ###$P < 0.001$ versus olive oil + PBS groups.
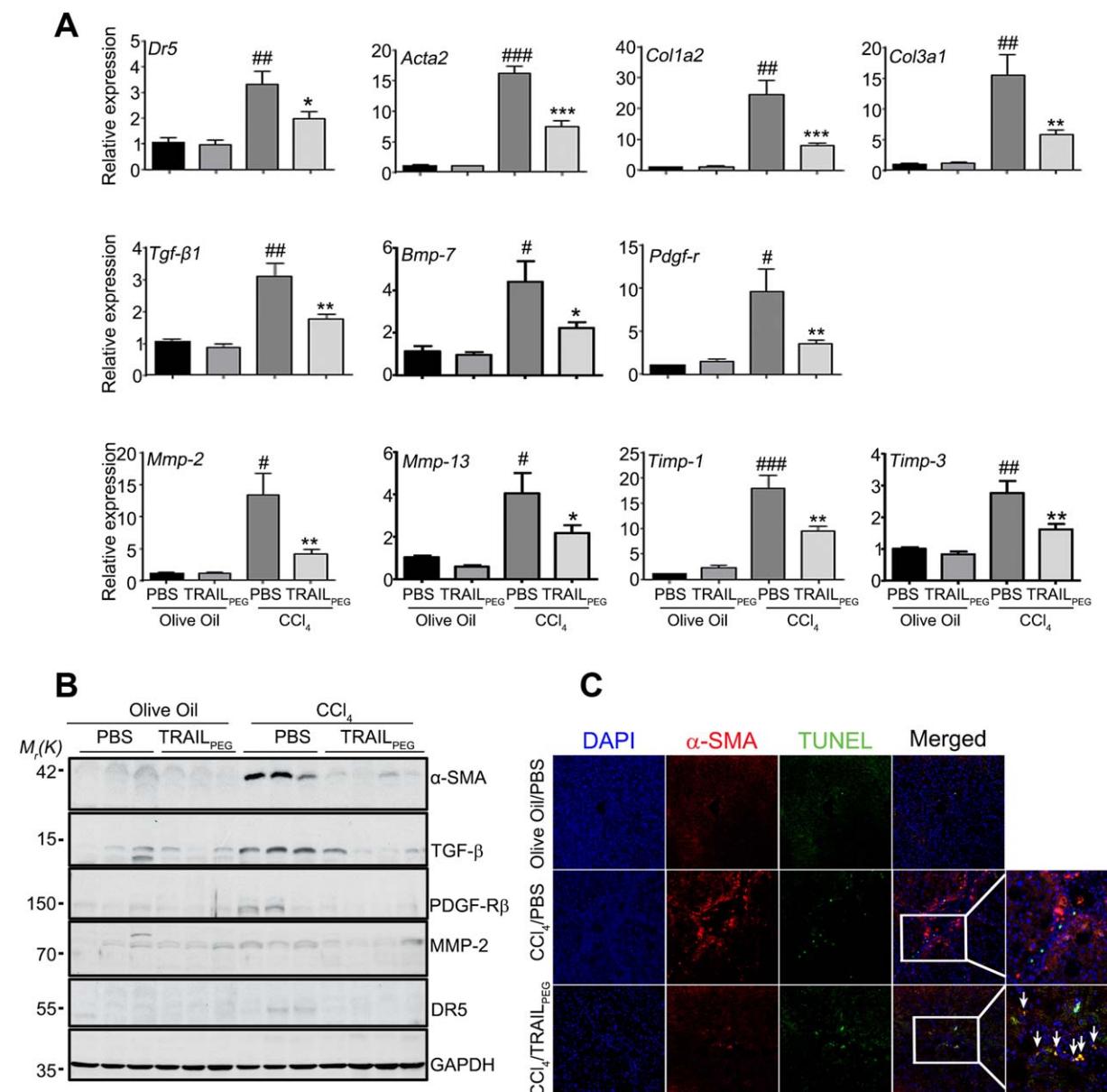
FIG. 3. Intravenously injected TRAIL$_{PEG}$ effectively targets aHSCs and simultaneously down-regulates multiple fibrogenic components during liver fibrogenesis. (A) Down-regulated gene expression profiles of TRAIL receptor (DR5), HSC activation, and fibrogenic markers from TRAIL$_{PEG}$-treated fibrotic livers (n = 4-10). Data are expressed as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus olive oil + PBS groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus CCl$_4$ + PBS groups. (B) Western blot analyses of rodent TRAIL receptor (DR5) and α-SMA expression, representative of HSC activation, along with other fibrogenic markers. (C) Immunofluorescence micrographs of liver sections from control (olive oil–treated) or chronic CCl$_4$ treatment with or without TRAIL-$_{PEG}$ stained for nuclei (DAPI, blue), aHSCs (α-SMA, red), apoptosis (TUNEL, green), and merged image (original magnification ×100). Arrows indicate TRAIL$_{PEG}$-induced apoptosis in aHSCs as well as overlap of TUNEL and α-SMA staining. Additional markers in the sera of rats are shown in Supporting Figure 3.

Fig. 4B, PBS-treated cirrhotic livers revealed advanced development of fibrosis such as nodule formation, demonstrated by intensely stained α-SMA–positive areas and collagen depositions. On the other hand, rats that had cirrhosis and were exposed to systemic TRAIL$_{PEG}$ showed a clear difference in macroscopic morphological appearance along with a marked reduction of α-SMA–positive areas and collagen deposition,
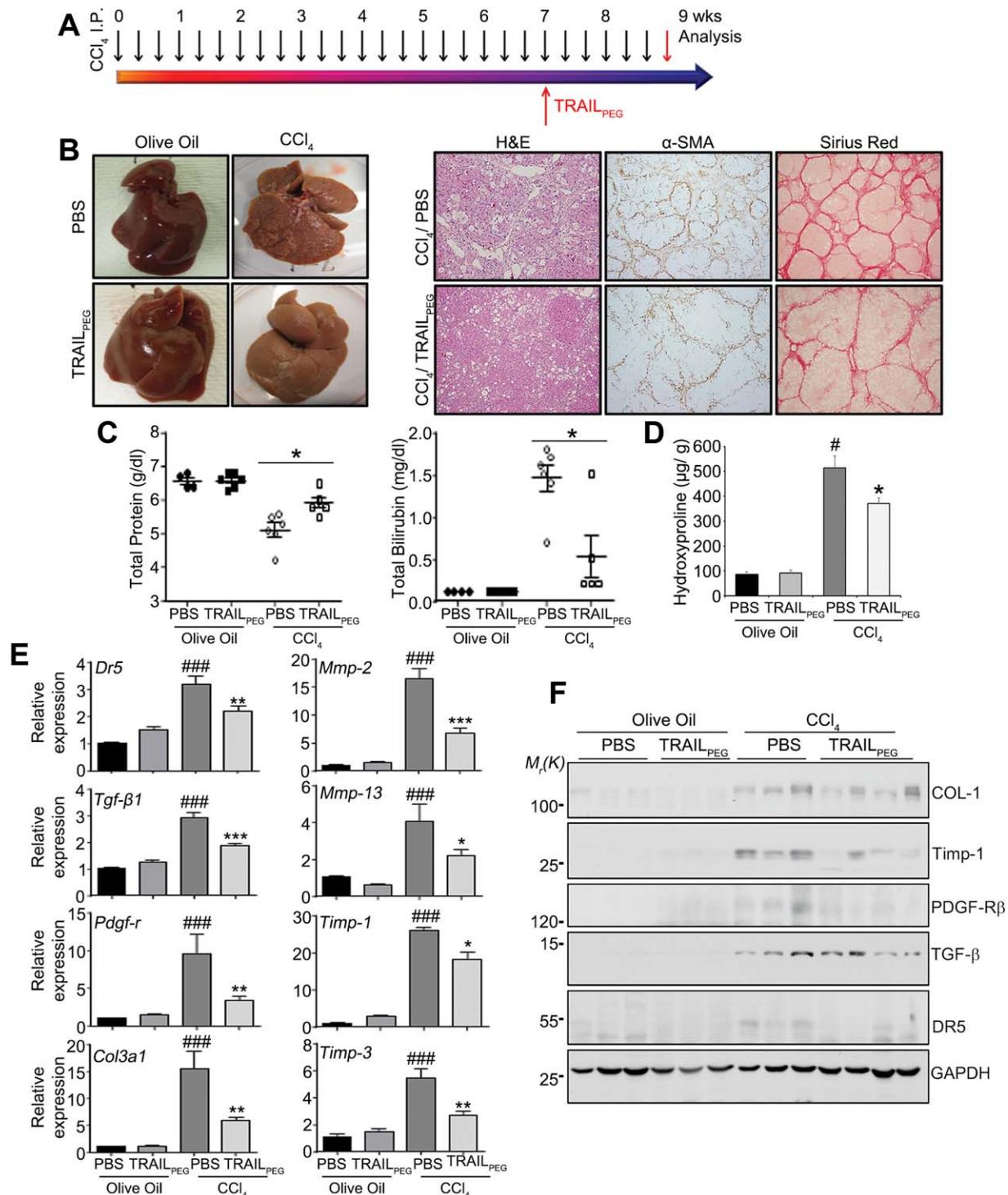
FIG. 4. Intravenously injected TRAIL$_{PEG}$ ameliorates CCl$_4$-induced liver cirrhosis in rats. (A) Intravenous TRAIL$_{PEG}$ (4 mg/kg) was administered daily for 2 weeks in control and rat models of CCl$_4$-treated (three times a week) liver cirrhosis. TRAIL$_{PEG}$ or PBS treatment was initiated at day 50 in control (olive oil–treated) and CCl$_4$-treated rats. Livers and blood samples were obtained at day 65. (B) Representative photographs of normal and cirrhotic livers from rats (left) and representative photomicrographs of liver sections stained with hematoxylin and eosin (H&E) and immunohistochemistry of activated HSC marker (α-SMA) and collagen deposition (sirius red) treated with or without TRAIL$_{PEG}$, according to the timeline in panel A, at day 65 (original magnification ×40). (C) Serum levels of total protein and bilirubin. (D) Quantification of collagen by hydroxyproline analysis in total livers (n = 4-6). (E) Gene expression profiles of TRAIL receptor (DR5), HSC activation markers, and fibrogenic markers from TRAIL$_{PEG}$- or PBS-treated cirrhotic livers as well as TRAIL$_{PEG}$- or PBS-treated normal livers (n = 4-6). Data are expressed as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus olive oil + PBS groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus CCl$_4$ + PBS groups. (F) Western blot analyses of TRAIL receptor (DR5) and fibrogenic markers. PBS-treated normal (olive oil–treated) liver samples are the control. Additional markers in the sera of rats and gene expression profiles are shown in Supporting Figure 6B.

as evidenced by immunohistochemistry analysis (Fig. 4B, Supporting Fig. 6A). TRAIL$_{PEG}$-treated cirrhotic animal models had increased serum levels of total protein and albumin with significantly lower total bilirubin, direct bilirubin, and hydroxyproline levels (Fig. 4C,D and Supporting Fig. 6B) than untreated animals with cirrhosis. At the mRNA and protein levels, TRAIL$_{PEG}$ treatment resulted in substantially down-regulated molecules associated with fibrogenesis (Fig. 4E,F and Supporting Fig. 6C). In TRAIL$_{PEG}$-treated cirrhotic rats, the relative fold decrease of multiple genes compared with control, including Dr5, Tgf-β1, Timp-1, Timp-3, Col3a1, Pdgf-r, Mmp-2, Mmp-13, and Bmp-7, was more pronounced than in TRAIL$_{PEG}$-treated fibrotic livers. Taken together, these data suggest that systemically delivered TRAIL$_{PEG}$, at a modest dose, histologically and functionally reverses CCl$_4$-induced liver cirrhosis to early-stage fibrosis.

TRAIL$_{PEG}$-INDUCED APOPTOSIS IN PRIMARY HUMAN HSCS

To investigate the mechanism of TRAIL sensitization in HSCs, changes in TRAIL signaling and TRAIL resistance-related components were studied by analyzing apoptotic and anti-apoptotic proteins *in vitro*. After we confirmed that up-regulation of DRs in immortalized LX2 human HSC cell lines (Supporting Fig. 7) was consistent with previous reports,[14] we tested TRAIL-induced apoptosis in primary human HSCs. Human HSCs were cultured for 2 days (quiescent) and 7 or 10 days (activated). Culture-activated HSCs (day 7 and 10) showed morphological changes and distinct induction of fibrogenic markers, DRs, and decoy receptors (Dcr1 and Dcr2) compared with qHSCs (day 2) at the mRNA and protein levels (Fig. 5A,B and Supporting Fig. 8A). These changes correlated with cell surface staining for functional DR5 and DR4 in aHSCs (Supporting Fig. 8B). During activation, HSCs deplete the antiapoptotic protein, XIAP, and augment the proapoptotic protein, BAK (Supporting Fig. 9A), which could contribute to the increased sensitivity of aHSCs to TRAIL-induced cell death (Fig. 5C,D). Interestingly, however, HSCs also highly up-regulate a series of antiapoptotic proteins such as BCL-2, BCL-XL, MCL-1, c-IAP1 (Supporting Fig. 9A,B) as well as c-FLIP, all of which are well known for inhibiting TRAIL-induced apoptosis in various cancer cells (Fig. 5E). In addition, receptor-activating protein 1, a kinase involved in necroptosis (pro-

grammed necrosis),[24] was up-regulated in aHSCs (Fig. 5E). Despite increased expression of antiapoptotic proteins, TRAIL$_{PEG}$ (1 μg/mL) treatment rapidly induced apoptosis in aHSCs in 3 hours as indicated by highly expressed apoptotic modulator levels, including DRs, cleaved PARP-1, and caspase-3.[25] Bright-field microscopy images of HSCs and results from cell death assays indicate that HSCs became highly sensitive to TRAIL$_{PEG}$ during activation (Fig. 5C,D and Supporting Fig. 9C). Based on gene knockdown studies, we demonstrate that TRAIL$_{PEG}$-induced apoptosis in aHSCs is mediated by either DR4 or DR5 (Fig. 5F). We also validated that activated primary HSCs are resistant to apoptosis when incubated with conventional toxic agents such as doxorubicin, cisplatin, H$_2$O$_2$, as well as in serum deprivation for 24 hours, but not against TRAIL$_{PEG}$ (Supporting Fig. 4E). We subsequently assessed DISC immunoprecipitation with DR4 and DR5 antibodies in HSCs and confirmed that TRAIL$_{PEG}$-induced DISC formation comprised caspase 8 and FADD in aHSCs only (Fig. 6A). Downstream, these complexes activate caspase-8 and thereby trigger extrinsic apoptosis. Caspase-8, FLIP, DR4, and DR5 were detected in DISC after exposure of aHSCs to FLAG-TRAIL, verifying that TRAIL-induced aHSC apoptosis is a result of caspase-8 activation in DISC initiated by triggered DR4/DR5 (Supporting Fig. 10). To determine whether TRAIL$_{PEG}$ induces apoptosis via a caspase-dependent pathway or necroptosis pathway, aHSCs were incubated with the caspase-8 inhibitor, z-IETD-fmk, and the pan-caspase inhibitor, Z-VAD-fmk and treated with TRAIL$_{PEG}$. Western blot analysis data, microscopic images, and MTT assays corroborate that the treatment of caspase-8 and pan-caspase inhibitors blocked TRAIL-induced apoptosis (Fig. 6B-D).

Discussion

By addressing the known limitations of TRAIL agonists in previous clinical studies and validating TRAIL receptors as a target in fibrosis, we demonstrate that TRAIL receptor agonists can have a significant therapeutic role in liver fibrosis and cirrhosis. Here, we targeted and eradicated the major originator of liver fibrosis, aHSCs, using an engineered TRAIL agonist as an antifibrotic agent (Fig. 6E). Liver fibrosis is the excess accumulation of ECM as a result of chronic inflammation induced by multiple causes, including alcoholic steatohepatitis, viral hepatitis,
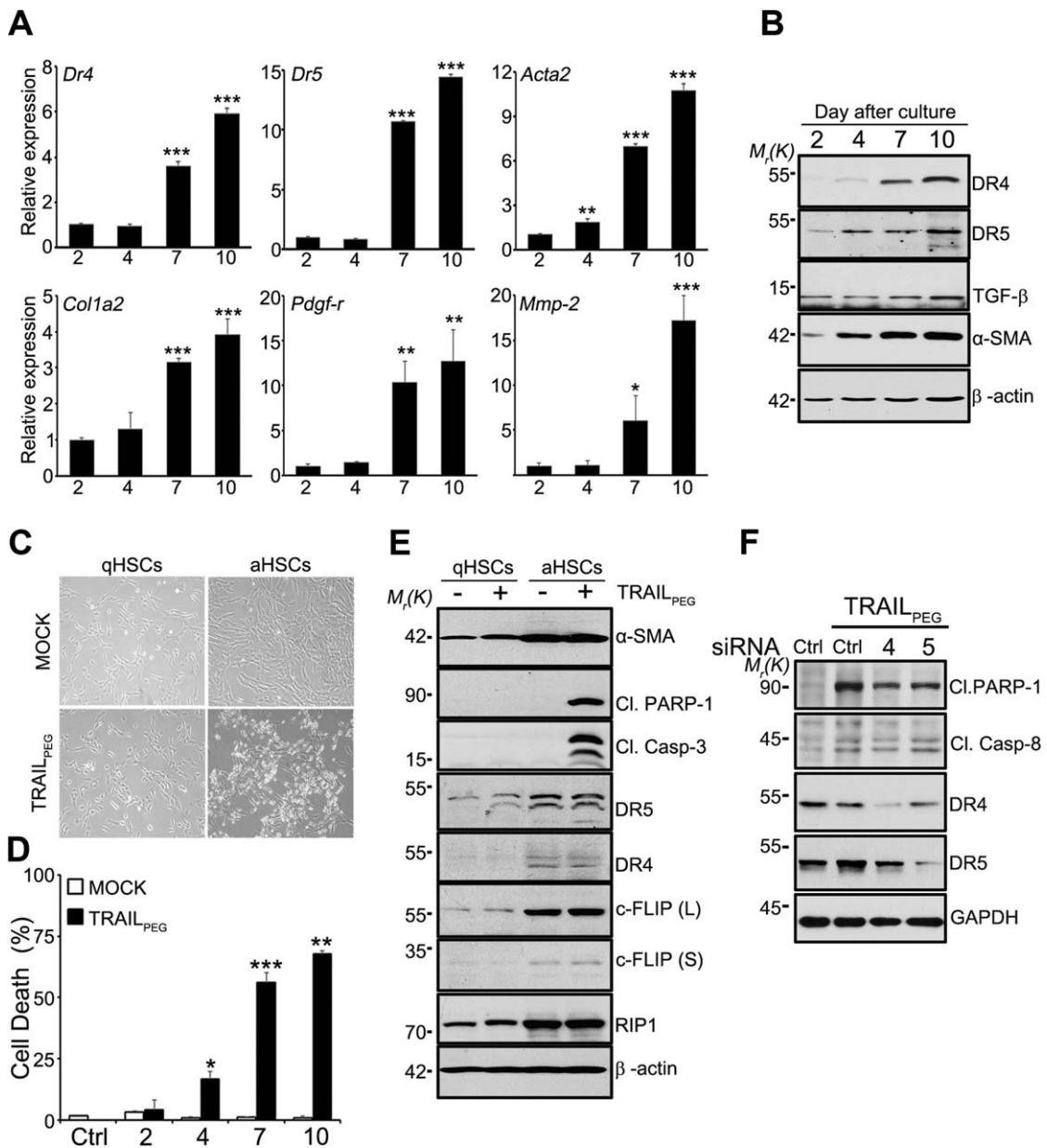
FIG. 5. HSCs up-regulate fibrogenic components and death receptors during activation and become sensitive to TRAIL_PEG-induced apoptosis. Human primary HSCs were culture-activated for various time points (days 2-10) and treated with TRAIL_PEG or vehicle followed by qRT-PCR or western blot analyses. (A) qRT-PCR of aHSC markers and TRAIL receptors during transition from the quiescent (day 2) to the culture-activated phenotype (days 4, 7, and 10). (B) Western blot analyses of antiapoptotic and proapoptotic markers in primary human HSCs during transition from the quiescent (day 2 in culture) to an activated phenotype (days 4, 7, and 10). α-SMA was used as an activation biomarker for HSCs. (C) Representative photos of qHSCs (day 2) and aHSCs (day 7) treated with TRAIL_PEG (1 μg/mL) for 3 hours. (D) Quantified TRAIL_PEG-induced cell death during HSC activation (days 2-10) analyzed by way of MTT assay. Data are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus qHSCs (day 2) (A) or MOCK (0.1% DMSO treatment) (D). (E) Western blot analysis of apoptotic markers and TRAIL receptors from qHSCs (day 2) and aHSCs (day 7) treated with vehicle or TRAIL_PEG (1 μg/mL) for 3 hours. (F) Western blot analysis of apoptotic markers from LX-2 cells transfected with control siRNA (Ctrl), DR4 siRNA, or DR5 siRNA and treated with TRAIL_PEG (1 μg/mL) for 8 hours.
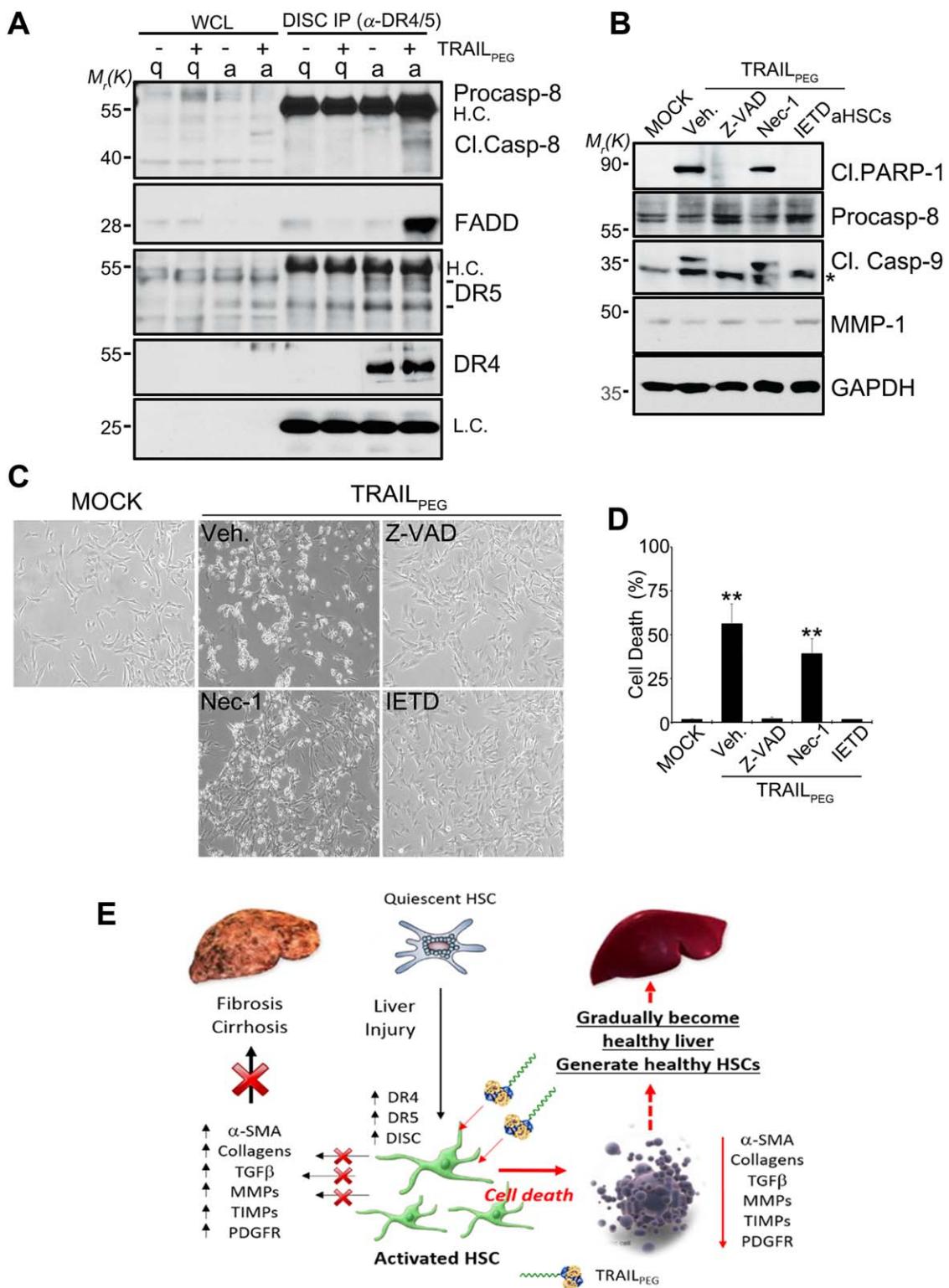
FIG. 6. TRAIL_PEG induces apoptosis in aHSCs, but not quiescent HSCs (qHSCs), through DISC and caspase-dependent pathway. (A) DISC immunoprecipitation studies. Western blot analysis of qHSCs (q, 2 days) and aHSCs (a, 7 days) treated with TRAIL_PEG (2 μg/mL, 60 minutes) and then immunoprecipitated with anti-DR4 and anti-DR5 antibodies. Whole cell lysates (WCL) are indicated (left). H.C., heavy chain; L.C. light chain. (B) Western blot analysis of apoptotic markers from aHSCs cotreated with TRAIL-_PEG (1 μg/mL) and various inhibitors of pan-caspase (Z-VAD, 20 μM), caspase-8 (IETD, 20 μM), and necroptosis (Nec-1, 50 μM) for 3 hours. (C) Representative photos of aHSCs cotreated with TRAIL_PEG (1 μg/mL) and various inhibitors of pan-caspase (Z-VAD, 20 μM), caspase-8 (IETD, 20 μM), and necroptosis (Nec-1, 50 μM) for 3 hours. (D) Quantified cell death analyses by MTT assay. Data are expressed as the mean ± SD. *P < 0.05, **P < 0.01 versus MOCK (0.1% DMSO treatment) (D). (E) Summarized effect of TRAIL_PEG on HSC activation and liver fibrogenesis.

nonalcoholic steatohepatitis, metabolic disorders, and autoimmune diseases. Even after elimination of the causative trigger, persistent chronic damage may result in extensive scarring in the tissue and a steep decline of potential reversibility. This can lead to cirrhosis and vasculature distortion, which causes liver failure, portal hypertension, hepatocellular carcinoma and premature death. Because liver fibrosis patients may be asymptomatic for decades and typically seek treatment only at late stages of fibrosis or cirrhosis,[26] preventative strategies during early stages of fibrosis may not always be clinically relevant. Therefore, antifibrotic drugs that prevent liver fibrosis progression toward cirrhosis or induce regression of advanced fibrosis and cirrhosis are needed urgently.

By nature, aHSCs are a major target for antifibrotic therapy, because they are the primary ECM-producing cells in the liver and orchestrate liver fibrogenesis. Therefore, inhibition of HSC activation or removal of aHSCs is a rational strategy for liver fibrosis therapy. A few studies that alter HSC activation by targeting intracellular signaling molecules modulating HSC activation[27] or inhibiting extracellular fibrogenic components have been reported.[6,28] As an alternative, our strategy selectively removes aHSCs and hence downregulates downstream fibrogenic signaling by facilitating targeted aHSC death while leaving normal hepatic cells and regenerated qHSCs unharmed. By targeting the TRAIL-induced cell death pathway in aHSCs *in vivo*, we demonstrate a promising antifibrotic strategy that may have broader implications toward drug development in diverse fibrotic diseases.

Although aHSCs do not undergo spontaneous apoptosis and are resistant to various proapoptotic stimuli,[7] including conventional cytotoxic agents (Supporting Fig. 9D), we confirm that aHSCs are highly sensitive to TRAIL-induced apoptosis (Fig. 5C-E). Notably, unlike recombinant TRAIL[11] and certain TRAIL receptor agonists[29] investigated for cancer, our engineered TRAIL did not induce apoptosis in off-target cells, including qHSCs or other normal cells such as hepatocytes (Fig. 1B,C and Supporting Fig. 1). The potential of using recombinant TRAIL as an antifibrotic agent was suggested by Taimr et al.[14] when they first demonstrated TRAIL-induced cell death in aHSCs in LX2 cell lines. However, limited studies demonstrate TRAIL as a potential strategy for aHSC eradication *in vitro* and only speculate at *in vivo* therapeutic activity. We recently showed that systemic administration of hyaluronic acid-conjugated TRAIL can prevent fibrosis in a mild model of fibrosis in rats,[30] but TRAIL-induced HSC cell death was not

established. To date, there are no studies that demonstrate antifibrotic efficacy of TRAIL in established fibrosis and cirrhosis animal models.

Recombinant TRAIL has an extremely short half-life, is unstable, and aggregates at high concentrations; therefore, unmodified TRAIL is limited for drug development. More than 10 PEGylated biologics are approved to date,[31] and over 20 PEGylated therapies are being investigated in the clinic,[32] demonstrating that PEGylation is an effective and—to the best of our knowledge—safe method to improve protein drug delivery. In addition, PEGylated proteins are considered less immunogenic than their non-PEGylated counterparts.[17] Some clinical concerns have been raised for high molecular weight PEGylated protein drugs, but clinical doses are drastically lower than those demonstrated for PEG toxicity.[33] TRAIL$_{PEG}$ overcomes the inherent limitations of TRAIL by improving its stability, extending its circulating half-life and reducing its aggregation while retaining its intrinsic biological activity (Fig. 1A-C).[15] In this study, we also defined up-regulation of DRs on aHSC/myofibroblasts in the parenchyma, α-SMA positive myofibroblasts, and inflammatory cells in fibrotic septa of human livers with alcoholic or viral cirrhosis (Supporting Fig. 2). The effects of site-specific PEGylation further enabled the *in vivo* investigation of TRAIL. We assessed the antifibrotic effects of TRAIL$_{PEG}$ in animal models of liver fibrosis and cirrhosis induced by CCl_4 administration (Figs. 2-4). Our results demonstrate that systemic administration of TRAIL$_{PEG}$ significantly ameliorates liver fibrosis and cirrhosis in animal models, as confirmed by reduced α-SMA and collagen deposition levels along with down-regulated expression of multiple fibrotic components in liver tissues (Figs. 2-4).

The concurrent down-regulation of fibrotic markers is a resounding characteristic of the TRAIL$_{PEG}$-treated fibrotic and cirrhotic animals, and we verify that it is predominantly a result of TRAIL-induced aHSC apoptosis *in vivo*. The levels of profibrogenic markers TGF-β and PDGF-R were significantly reduced in the TRAIL$_{PEG}$-treated group in both fibrotic and cirrhotic animal models (Figs. 3A,B, and 4F,G). TGF-β and PDGF-R are typically produced by platelets and Kupffer cells/macrophages during the early stages of liver damage; however, after activation of HSCs, aHSCs become the major source of these cytokines, which act in an autocrine manner to promote further HSC activation, cell migration, and ECM formation.[28,34] In addition to diminished levels

of TGF-β and PDGF-R in the TRAIL$_{PEG}$-treated group, there was decreased expression of collagens, matrix metalloproteinases, and TIMPs in fibrotic and cirrhotic livers (Figs. 3 and 4). Therapeutic antibodies or small molecule inhibitors targeting individual fibrogenic molecules have been investigated as antifibrotic agents.[6] Instead of independently targeting one of many fibrogenic components such as TGF-β, PDGF-R, matrix metalloproteinases, or TIMPS, the TRAIL$_{PEG}$ treatment strategy introduced here simultaneously inhibits all of their activities. Using this single agent, we demonstrate that depleting the significant originator cell of fibrosis, aHSCs, is a viable and powerful therapeutic strategy for reversing liver fibrosis and cirrhosis. In addition, based on blood chemistry and histology, TRAIL$_{PEG}$ did not induce any noticeable toxicity *in vivo*—including in the liver—after repetitive injections.

Immortalized LX2 cells and activated primary human HSCs demonstrated a significant up-regulation of DRs and underwent TRAIL-induced apoptosis (Fig. 5 and Supporting Figs. 7-10). To investigate the mechanisms of TRAIL sensitization in HSCs, changes in TRAIL signaling and TRAIL resistance were studied by analyzing apoptotic and antiapoptotic proteins in both primary human HSCs and LX2 cells. Interestingly, we discovered that aHSCs up-regulate not only DRs but also multiple antiapoptotic proteins that are well known to strongly inhibit TRAIL-induced apoptosis in various cancer cells such as BCL-2, BCL-XL, MCL-1, and FLIP (Fig. 5E and Supporting Fig. 9). In the field of oncology, extensive studies have demonstrated that TRAIL resistance in cancer cells can be overcome by using inhibitors of antiapoptotic proteins to potentiate apoptosis.[35,36] However, we did not observe TRAIL resistance in aHSCs. Based on our molecular analysis, apoptosis is the main cell death pathway in aHSCs treated with TRAIL$_{PEG}$. However, we also show evidence that necroptosis may be involved in TRAIL$_{PEG}$-induced cell death, based on the slight reduction in cell death over control when HSCs were treated with TRAIL$_{PEG}$ and necroptosis inhibitor, Nec-1 (Fig. 6D) as well as the increase in receptor-activating protein 1 expression during HSC activation (Fig. 5E). Because TRAIL-induced apoptosis has been predominantly investigated in cancer cells and not in primary human HSCs, we are currently exploring mechanisms of TRAIL resistance and sensitivity by comparing TRAIL death pathways between HSCs and cancer cells.[37] TRAIL$_{PEG}$ could also target cirrhosis-

associated hepatocellular carcinoma when combined with an appropriate TRAIL sensitizer. Further investigation is needed to explore the synergistic effect of TRAIL$_{PEG}$ to eradicate liver tumor cells while simultaneously reversing cirrhosis. Furthermore, the effect of TRAIL$_{PEG}$ on other types of cells that play a key role in liver fibrosis must be elucidated. During liver fibrosis, hepatic resident cells including liver sinusoidal endothelial cells, Kupffer cells and injured hepatocytes contribute to HSC activation by producing distinct cytokines and growth factors. It is conceivable that such cells can be targeted by systemically administered TRAIL$_{PEG}$ and may be partly responsible for the antifibrotic effect of TRAIL-based therapy. *In vivo* resistance or sensitivity of hepatic resident cells against systemically administered TRAIL during liver injuries must be clearly elucidated. Lastly, to fully understand the role of antifibrotic efficacies of TRAIL$_{PEG}$ in a preclinical setting, the drug should be evaluated in mechanistically distinct animal models of human hepatic fibrosis. Overall, further studies into the role of TRAIL signaling in aHSCs, the effect of TRAIL$_{PEG}$ on hepatic resident cells during liver fibrosis *in vivo*, and antifibrotic activity in additional fibrotic animal models is required. Our studies in severe CCl$_4$-induced fibrosis and cirrhosis rat models warrant the further development of TRAIL$_{PEG}$ for human liver fibrosis.

We introduce a new strategy to ameliorate liver fibrosis and cirrhosis *in vivo* by targeting DR-expressing aHSCs through systemically administered, long-acting TRAIL agonist, TRAIL$_{PEG}$. Our studies identify up-regulated TRAIL receptors in aHSCs as an *in vivo* clinical target to treat liver fibrosis and cirrhosis and validate an intravenously administered recombinant TRAIL agonist as a promising antifibrotic agent. Combined with the high unmet clinical need for effective antifibrotic therapies, our study warrants clinical translation of TRAIL-based therapies for liver fibrosis and potentially other fibrotic disorders.

REFERENCES

1) Friedman SL, Sheppard D, Duffield JS, Violette S. Therapy for fibrotic diseases: nearing the starting line. Sci Transl Med 2013; 5:167sr1.
2) Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115: 209-218.

3) Altamirano J, Bataller R. Alcoholic liver disease: pathogenesis and new targets for therapy. Nat Rev Gastroenterol Hepatol 2011;8:491-501.

4) Friedman SL. Evolving challenges in hepatic fibrosis. Nat Rev Gastroenterol Hepatol 2010;7:425-436.

5) Friedman SL. Hepatic stellate cells. Prog Liver Dis 1996;14: 101-130.

6) Schuppan D, Kim YO. Evolving therapies for liver fibrosis. J Clin Invest 2013;123:1887-1901.

7) Novo E, Marra F, Zamara E, Valfre di Bonzo L, Monitillo L, Cannito S, et al. Overexpression of Bcl-2 by activated human hepatic stellate cells: resistance to apoptosis as a mechanism of progressive hepatic fibrogenesis in humans. Gut 2006;55:1174-1182.

8) Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 1999;5: 157-163.

9) Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 2008;8:782-798.

10) Soria JC, Mark Z, Zatloukal P, Szima B, Albert I, Juhasz E, et al. Randomized phase II study of dulanermin in combination with paclitaxel, carboplatin, and bevacizumab in advanced non-small-cell lung cancer. J Clin Oncol 2011;29:4442-4451.

11) Lemke J, von Karstedt S, Zinngrebe J, Walczak H. Getting TRAIL back on track for cancer therapy. Cell Death Differ 2014;21:1350-1364.

12) Kelley SK, Harris LA, Xie D, Deforge L, Totpal K, Bussiere J, et al. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. J Pharmacol Exp Ther 2001;299:31-38.

13) Ashkenazi A, Holland P, Eckhardt SG. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol 2008;26: 3621-3630.

14) Taimr P, Higuchi H, Kocova E, Rippe RA, Friedman S, Gores GJ. Activated stellate cells express the TRAIL receptor-2/death receptor-5 and undergo TRAIL-mediated apoptosis. HEPATOLOGY 2003;37:87-95.

15) **Chae SY**, **Kim TH**, Park K, Jin CH, Son S, Lee S, et al. Improved antitumor activity and tumor targeting of NH(2)-terminal-specific PEGylated tumor necrosis factor-related apoptosis-inducing ligand. Mol Cancer Ther 2010;9:1719-1729.

16) Kim TH, Youn YS, Jiang HH, Lee S, Chen X, Lee KC. PEGylated TNF-related apoptosis-inducing ligand (TRAIL) analogues: pharmacokinetics and antitumor effects. Bioconjug Chem 2011; 22:1631-1637.

17) Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov 2003;2:214-221.

18) Kang JS, Deluca PP, Lee KC. Emerging PEGylated drugs. Expert Opin Emerg Drugs 2009;14:363-380.

19) Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, et al. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat Med 2001;7:383-385.

20) **Ganten TM**, **Koschny R**, Sykora J, Schulze-Bergkamen H, Buchler P, Haas TL, et al. Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. Clin Cancer Res 2006;12:2640-2646.

21) Nair PM, Flores H, Gogineni A, Marsters S, Lawrence DA, Kelley RF, et al. Enhancing the antitumor efficacy of a cell-surface death ligand by covalent membrane display. Proc Natl Acad Sci U S A 2015;112:5679-5684.

22) Malhi H, Barreyro FJ, Isomoto H, Bronk SF, Gores GJ. Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. Gut 2007;56:1124-1131.

23) Constandinou C, Henderson N, Iredale JP. Modeling liver fibrosis in rodents. Methods Mol Med 2005;117:237-250.

24) Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 2010;11:700-714.

25) Jin ZY, El-Deiry WS. Overview of cell death signaling pathways. Cancer Biol Ther 2005;4:139-163.

26) Friedman SL. Liver fibrosis—from bench to bedside. J Hepatol 2003;38:S38-S53.

27) Bartneck M, Warzecha KT, Tacke F. Therapeutic targeting of liver inflammation and fibrosis by nanomedicine. Hepatobiliary Surg Nutr 2014;3:364-376.

28) Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. HEPATOLOGY 2015;61:1066-1079.

29) Papadopoulos KP, Issacs R, Bilic S, Kentsch K, Huet HA, Hofmann M. et al. Unexpected hepatotoxicity in a phase I study of TAS266, a novel tetravalent agonistic Nanobody targeting the DR5 receptor. Cancer Chemother Pharmacol 2015;75:887-895.

30) Yang JA, Kong WH, Sung DK, Kim H, Kim TH, Lee KC, et al. Hyaluronic acid-tumor necrosis factor-related apoptosis-inducing ligand conjugate for targeted treatment of liver fibrosis. Acta Biomater 2015;12:174-182.

31) Alconcel SNS, Baas AS, Maynard HD. FDA-approved poly(ethylene glycol)-protein conjugate drugs. Polym Chem 2011;2:1442-1448.

32) Swierczewska M, Lee KC, Lee S. What is the future of PEGylated therapies? Expert Opin Emerg Drugs 2015;20:531-536.

33) Webster R, Elliott V, Park BK. PEG and PEG conjugates toxicity: towards an understanding of the toxicity of PEG and its relevance to PEGylated biologicals. In: Veronese FM, ed. PEGylated Protein Drugs: Basic Science and Clinical Applications. Basel, Switzerland: Birkhauser Verlag; 2009:127-146

34) Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med 2012;18:1028-1040.

35) Hellwig CT, Rehm M. TRAIL signaling and synergy mechanisms used in TRAIL-based combination therapies. Mol Cancer Ther 2012;11:3-13.

36) Voelkel-Johnson C. TRAIL-mediated signaling in prostate, bladder and renal cancer. Nat Rev Urol 2011;8:417-427.

37) **Yin C**, **Evason KJ**, Asahina K, Stainier DY. Hepatic stellate cells in liver development, regeneration, and cancer. J Clin Invest 2013;123:1902-1910.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28432/suppinfo.