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Systemic PEGylated TRAIL treatment ameliorates liver cirrhosis in rats by eliminating activated hepatic stellate cells

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Abstract

Liver fibrosis is a common outcome of chronic liver disease and leads to liver cirrhosis and hepatocellular carcinoma. No FDA-approved targeted anti-fibrotic 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

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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 (DRs) 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. Here, we demonstrate that intravenous $TRAIL_{PEG}$ has a markedly extended half-life over native-type TRAIL in non-human 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. In conclusion, TRAIL-based therapies could serve as new therapeutics for liver fibrosis/cirrhosis and possibly other fibrotic diseases.

Keywords

apoptosis; cirrhosis; death receptors; fibrosis; hepatic stellate cells; TRAIL

INTRODUCTION

The mechanisms that underlie the pathogenesis of liver fibrosis have been extensively studied, 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 stellate cells (aHSCs) express platelet-derived growth factor (PDGF) and PDGF receptors (PDGF-Rs). PDGF induces HSC proliferation, resulting in increased production of pro-fibrogenic cytokines such as transforming growth factor-β (TGF-β), which further activate HSCs to up-regulate alpha 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 alpha

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(TNF-α), or DNA intercalating agents such as anticancer drugs and oxidative stress mediators.[7]

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type II 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 3/6/7, resulting in apoptosis. Another pathway of apoptosis is the induction of mitochondrial dysfunction and membrane permeabilization causing release of cytochrome c that activates caspase 9 and finally cleavage of caspase 3/7 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, recombinant human TRAIL, has been investigated as an anti-cancer therapy; but, its clinical efficacy was disappointing,[10, 11] probably due to TRAIL-resistance in primary cancer cells and the short half-life of the protein (less than 5 min in rodents and 30 min 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 fully investigated. 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. Herein, we determine if such a strategy has therapeutic potential in liver fibrosis and cirrhosis. We verified if TRAIL is a suitable target for anti-fibrotic 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 utilized 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, the Division of Pediatric Gastroenterology and Nutrition, University of Minnesota, Minneapolis, MN) provided frozen alcoholic cirrhotic liver samples and paraffin embedded liver samples from patients

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with HBV, HCV, ALD, or ALD/HCV with end-stage cirrhosis who received liver transplantation. Liver diseases were diagnosed by LTPDS and based on a history of alcohol drinking, infected viral markers and liver histology. Liver pathology of these specimens showed bridging fibrosis and cirrhosis. LTPDS also provided paraffin embedded normal healthy liver specimens obtained from human donor livers not used for transplantation. The LTPDS was funded by NIH Contract #N01-DK-9-2310. The protocol for using these liver samples has been approved by the LTPDS of the University of Minnesota and the National Institutes of Health. Frozen normal human liver tissues were purchased from TRL (Triangle Research Labs LLC, Durham, NC) for analysis of protein expression by western blot as controls.

Human primary hepatocyte culture and $TRAIL_{PEG}$ treatment

Cryopreserved human primary hepatocytes, human hepatocyte plating medium, and thawing medium were obtained from TRL (Triangle Research Labs, LLC, Durham, NC). 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 h. After cells were harvested, the expression of TRAIL receptors (DR4/DR5) and apoptosis markers were determined by Western blot analysis, and cell viability was analyzed by 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. Sprague Dawley (SD) male rats at the age of 5–6 weeks (BW 120–150 g) were purchased from Charles River (Germantown, MD). Rats were divided into 4 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 (i.p.) injection or olive oil as control groups for a total of 4 weeks. At day 29, rats were then treated with 4 mg/kg of $TRAIL_{PEG}$ via intravenous (i.v.) injection every day for ten days or 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 i.p. 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 $TRAIL_{PEG}$ via i.v. injection every day for fourteen days or 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 by a paired or unpaired t-test. Differences among multiple means were assessed, as indicated, by one-way ANOVA followed by Tukey's post-hoc test or by the Student's t-test as appropriate. Error bars represent s.d. or s.e.m., as indicated. Probabilities of $P < 0.05$ or as indicated were considered statistically significant.

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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 makes 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 half-life of protein drugs as well as reduce protein aggregation.[17,18] We demonstrated that $TRAIL_{PEG}$ has an improved pharmacodynamic (PD) profile in a tumor xenograft model and pharmacokinetic (PK) profile in rats compared to recombinant TRAIL.[15] $TRAIL_{PEG}$ demonstrated a substantially extended half-life (8.6 h) in cynomolgus monkeys after i.v. injection compared to His-iLZ-TRAIL (0.9 h) (Fig. 1A and Table S1). Native-type TRAIL is non-toxic to primary hepatocytes, because hepatocytes, like many non-transformed 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 5,000 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 (Fig. S1). Despite having a lower toxicity profile, $TRAIL_{PEG}$ maintained equal cancer killing efficiency when compared to 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 upregulated 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 hepatitis B virus (HBV), hepatitis C virus (HCV) infection, or chronic alcohol consumption (Fig. S2), and co-localized with α-SMA^+ aHSCs (Fig. 1D). Western blot analyses of liver tissues from patients with alcoholic cirrhosis showed strong up-regulation of DR5 and moderate

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upregulation of DR4 compared to healthy livers (Fig. 1E, 1F). 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 PK in non-human primates, we were motivated to explore $TRAIL_{PEG}$ and its ability to eradicate aHSC 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 treatment of CCl_4 in rats,[23] as shown in the treatment schedule in Fig. 2A. Two control groups received olive oil alone. After four weeks of CCl_4 exposure, fibrotic rats received $TRAIL_{PEG}$ (4 mg/kg, protein-based, at day 29) or PBS daily for a total of ten 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 (ALP) and total bilirubin (Fig. 2B) were significantly lower in sera from $TRAIL_{PEG}$ treated fibrotic rats than the PBS-treated fibrotic rats. Alanine transaminase and aspartate transaminase (ALT and AST, respectively) were not significantly different between $TRAIL_{PEG}$ and PBS treated groups, likely due to continuous CCl_4-induced liver damage during the study (Fig. S3). 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, 3E). 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 mRNA and protein levels. Quantitative real time PCR (qPCR) 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-beta 1), Bmp-7 (bone morphogenetic protein-7), Pdgf-r (platelet-derived growth factor receptors, PDGF-R), Mmp-2, Mmp-13 (matrix metalloproteinases-2 and -13), Timp-1, -3 (tissue inhibitor of metalloproteinase-1 and -3) and (Fig. 3A). Western blot analyses confirmed a significant decrease in expression levels of these proteins in the $TRAIL_{PEG}$-treated group (Fig. 3B, S4). 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 $\alpha\text{-SMA}^+$ aHSCs and apoptosis using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling). In control (olive oil-treated) healthy livers, no strong α-SMA and TUNEL staining was observed. In $TRAIL_{PEG}$-treated fibrotic livers, TUNEL staining co-localized with α-SMA, validating that apoptosis occurred in $\alpha\text{-SMA}^+$ aHSCs (Fig. 3C, S5). 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 ALT and AST (Fig. S3).

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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 fourteen 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. 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, cirrhotic rats 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 as evidenced by immunohistochemistry analysis (Fig. 4B, S6A). $TRAIL_{PEG}$ 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, 4D, S6B) than untreated cirrhotic animals. At the mRNA and protein levels, $TRAIL_{PEG}$ treatment resulted in substantially down-regulated molecules associated with fibrogenesis (Fig. 4E, 4F, S6C). In $TRAIL_{PEG}$-treated cirrhotic rats, the relative fold decrease of multiple genes compared to 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 hepatic stellate cells

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 up-regulation of DRs in immortalized LX2 human HSC cell lines (Fig. S7) consistent with previous reports,[14] we set out to test 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 to qHSCs (Day 2) at the mRNA and protein levels (Fig. 5A, 5B, S8A). These changes correlated with cell surface staining for functional DR5 and DR4 in aHSCs (Fig. S8B). During activation, HSCs deplete the anti-apoptotic protein, XIAP, and augment the pro-apoptotic protein, BAK (Fig. S9A), which could contribute to the increased sensitivity of aHSCs to TRAIL-induced cell death (Figs. 5C, 5D). Interestingly, however, activated HSCs also highly up-regulate a series of anti-apoptotic proteins such as BCL-2, BCL-XL, MCL-1, c-IAP1 (Figs. S9A, S9B) 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 (RIP1), a kinase involved in necroptosis (programmed necrosis),[24] was up-regulated in aHSCs (Fig. 5E). Despite increased expression of anti-apoptotic proteins, $TRAIL_{PEG}$ (1 μg/mL) treatment rapidly induced apoptosis in aHSCs in 3 hrs as indicated by highly expressed apoptotic modulator levels, including DRs, cleaved PARP-1, and caspase-3.[25] Bright-field microscopy images of HSCs

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and results from cell death assays indicate that HSCs became highly sensitive to $TRAIL_{PEG}$ during activation (Fig. 5C, 5D, S9C). 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_2O_2, as well as in serum deprivation for 24 hr, but not against $TRAIL_{PEG}$ (Fig. S4E). We subsequently assessed DISC immunoprecipitation (IP) with DR4 and DR5 antibodies in HSCs and confirmed that $TRAIL_{PEG}$-induced DISC formation comprised of 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 TRAIL-induced aHSC apoptosis is a results of caspase-8 activation in DISC initiated by triggered DR4/DR5 (Fig. S10). To determine if $TRAIL_{PEG}$ 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 target and eradicate 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, non-alcoholic steatohepatitis (NASH), 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 (HCC) 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 urgently needed.

By nature, aHSCs are a major target for antifibrotic therapy, because they are the primary ECM-producing cell in the liver and orchestrate liver fibrogenesis. Thus 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 down-regulates downstream fibrogenic signaling by facilitating targeted aHSC death while leaving normal hepatic cells and regenerated quiescent HSCs unharmed. By targeting the TRAIL-induced cell death

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pathway in aHSCs *in vivo*, we demonstrate a promising antifibrotic strategy that may have broader implications towards 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 (Fig. S9D), 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, 1C, S1). The potential of utilizing recombinant TRAIL as an antifibrotic agent has been suggested by Gores *et al.*, when they firstly demonstrated TRAIL-induced cell death in aHSCs in LX2 cell lines.[14] 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; thus, unmodified TRAIL has limited uses for *in vivo* drug development. More than ten PEGylated biologics are approved to date[31] and over twenty 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 respective 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 (Fig. S2). 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 (Fig. 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 (Fig. 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 (Fig. 3A, 3B, 4F, 4G). TGF-β and PDGF-R are typically produced by platelets and Kupffer cells/macrophages in early stages of liver damage; but 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

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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, MMPs and TIMPs in fibrotic and cirrhotic livers (Fig. 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, MMPs 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 demonstrate a significant up-regulation of DRs and undergo TRAIL-induced apoptosis (Fig. 5, S7–S10). To investigate the mechanisms of TRAIL sensitization in HSCs, changes in TRAIL signaling and TRAIL resistance were studied by analyzing apoptotic and anti-apoptotic proteins in both primary human HSCs and LX2 cells. Interestingly, we discovered that aHSCs up-regulate not only DRs but also multiple anti-apoptotic 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, S9). In the field of oncology, extensive studies demonstrate that TRAIL-resistance in cancer cells can be overcome by using inhibitors of anti-apoptotic 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 $TRAIL_{PEG}$. Yet, 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 RIP1 expression during HSC activation (Fig. 5E). Since 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 HCC 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

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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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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We thank Theraly Pharmaceuticals for providing $TRAIL_{PEG}$.

Abbreviations

CCl_4	carbon tetrachloride
qHSC	quiescent hepatic stellate cells
aHSC	activated hepatic stellate cells
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
$TRAIL_{PEG}$	PEGylated TRAIL
PEG	poly(ethylene glycol)
DR	death receptors
DcR	decoy receptor
ECM	extracellular matrix
α-SMA	alpha smooth muscle actin
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinase
TGF-β	transforming growth factor beta

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PDGF	platelet-derived growth factor
DISC	death inducing signaling complex
FADD	fas-associated death domain
Bcl-2	B cell lymphoma 2
Bcl-xL	B cell lymphoma-extra large
MCL-1	myeloid cell leukemia 1
PARP-1	poly (ADP-ribose) polymerase 1
c-FLIP	cellular (FADD-like IL-1β-converting enzyme)-inhibitory protein

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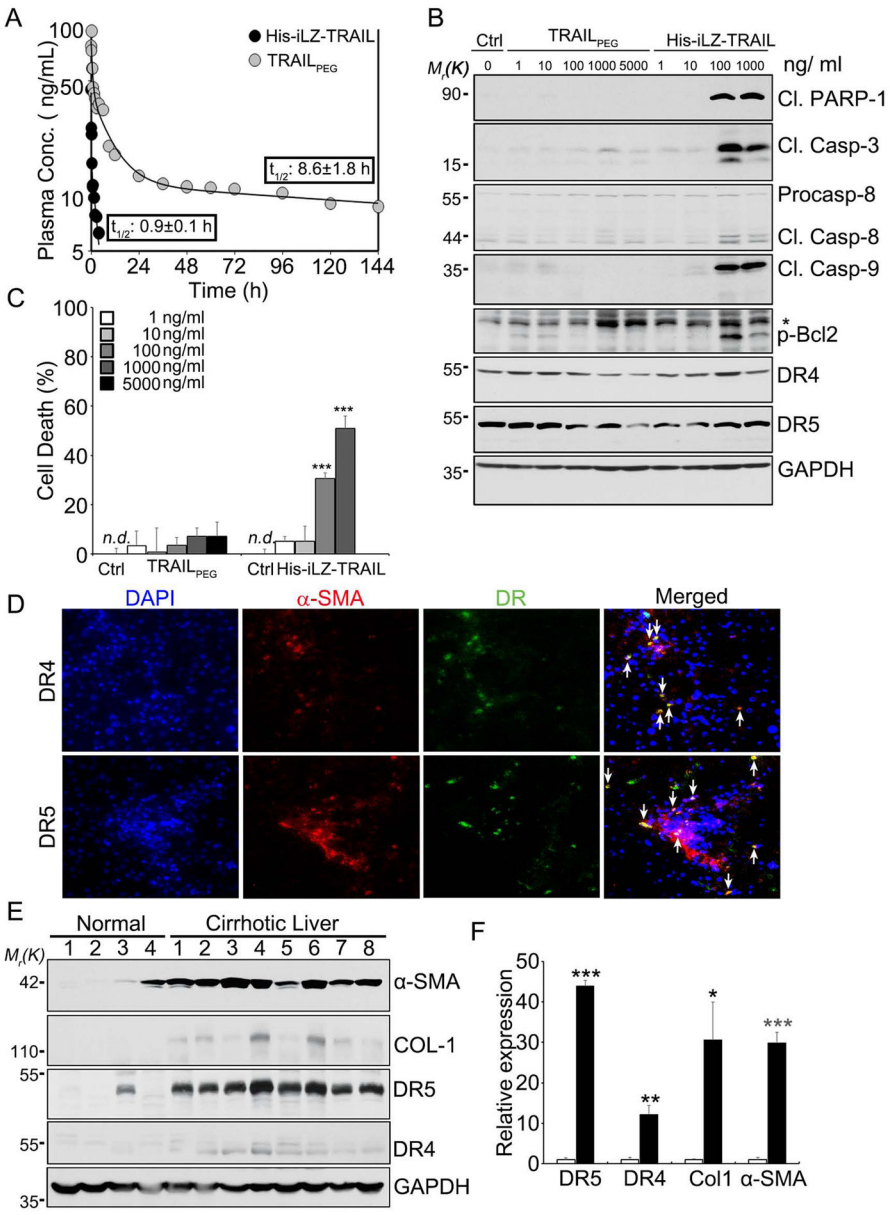

Figure 1. Intravenously injected TRAIL$_{PEG}$ shows extended half-life in non-human primates and no toxicity in primary human hepatocytes. TRAIL receptors, DR5 and DR4, are upregulated in human cirrhotic livers and co-localized in aHSCs

(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. Control (Ctrl) group is untreated. (B) Western blot analysis of hepatocytes treated with various concentrations of TRAIL$_{PEG}$ or His-iLZ-TRAIL for 3 h. (C) Quantified cell death analyzed by the MTT assay after treatment of primary hepatocytes with TRAIL$_{PEG}$ or His-iLZ-TRAIL. Data expressed as mean ± s.d. *** *P < 0.001* vs. non-treated groups (Ctrl). n.d., non-detectable. (D) Double immunofluorescence micrographs of cirrhotic liver sections stained for nuclei (DAPI, blue) with DR4 or DR5 (green) and α-SMA (red). Arrows indicate co-localized DR4 or DR5 with

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α-SMA-positive cells, aHSCs. Original magnification 200x. (E) Western blot analyses of human normal (4 human samples, lanes labeled 1–4) and alcoholic cirrhotic liver tissues (8 patient samples, lanes labeled 1–8) (ALC). (F) Densitometry analysis of western blots from (E) shown as relative protein expression normalized to healthy liver tissue. Data expressed as mean ± s.e.m $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. non-treated groups (Ctrl).

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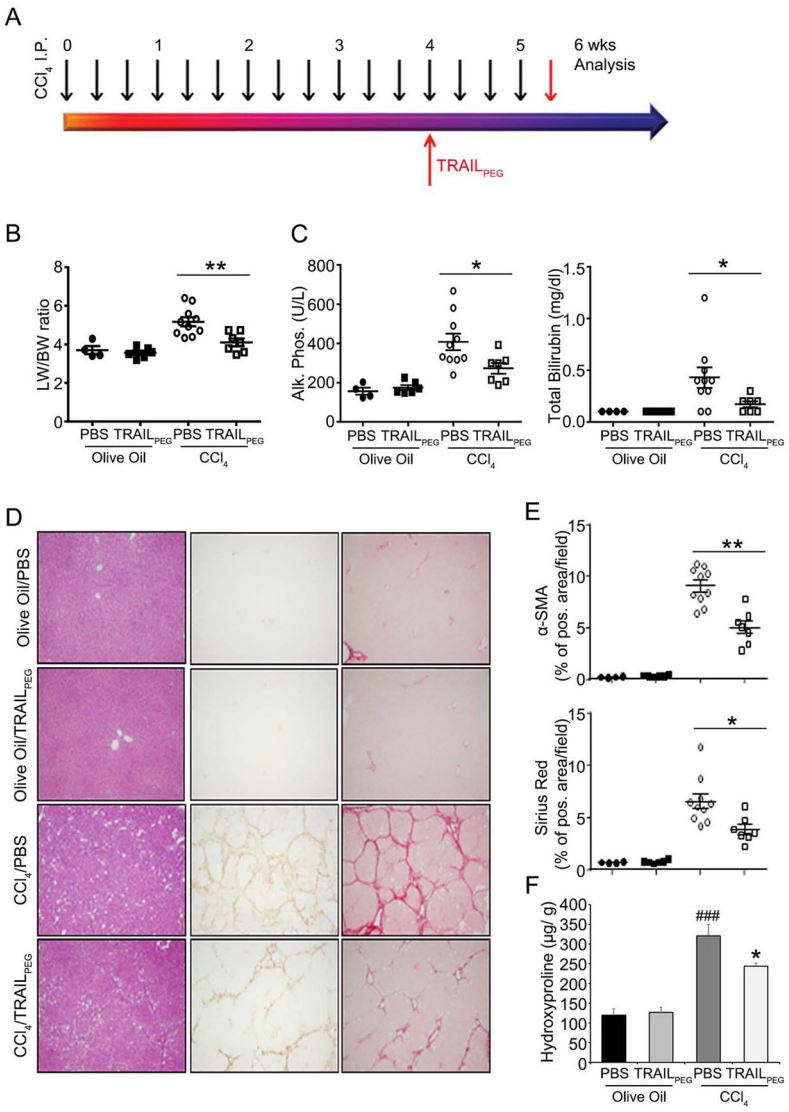

Figure 2. Intravenously injected $TRAIL_{PEG}$ ameliorates CCl_4-induced liver fibrosis in rats

(A) $TRAIL_{PEG}$ (4 mg/kg) was intravenously treated 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-treated rats. Livers and blood samples were obtained at day 40. (B) Liver weight (LW)/body weight (BW) analyses. (C) Serum levels of alkaline phosphatase (Alk. Phos.) and total bilirubin. (D) Representative photomicrographs of liver sections from control (olive oil-treated) or chronic CCl_4-treated rats with or without $TRAIL_{PEG}$ stained with hematoxylin and eosin (H&E) and immunohistochemistry of activated HSC marker (α-SMA) and collagen deposition (Sirius Red). Original magnification 40x. (E) Digital image quantification of α-SMA and Sirius Red (collagen) staining. (F) Quantification of collagen by hydroxyproline analysis in total livers. Data expressed as mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. CCl_4 + PBS groups. $^{\#\#\#}P < 0.001$ vs. olive oil + PBS groups.

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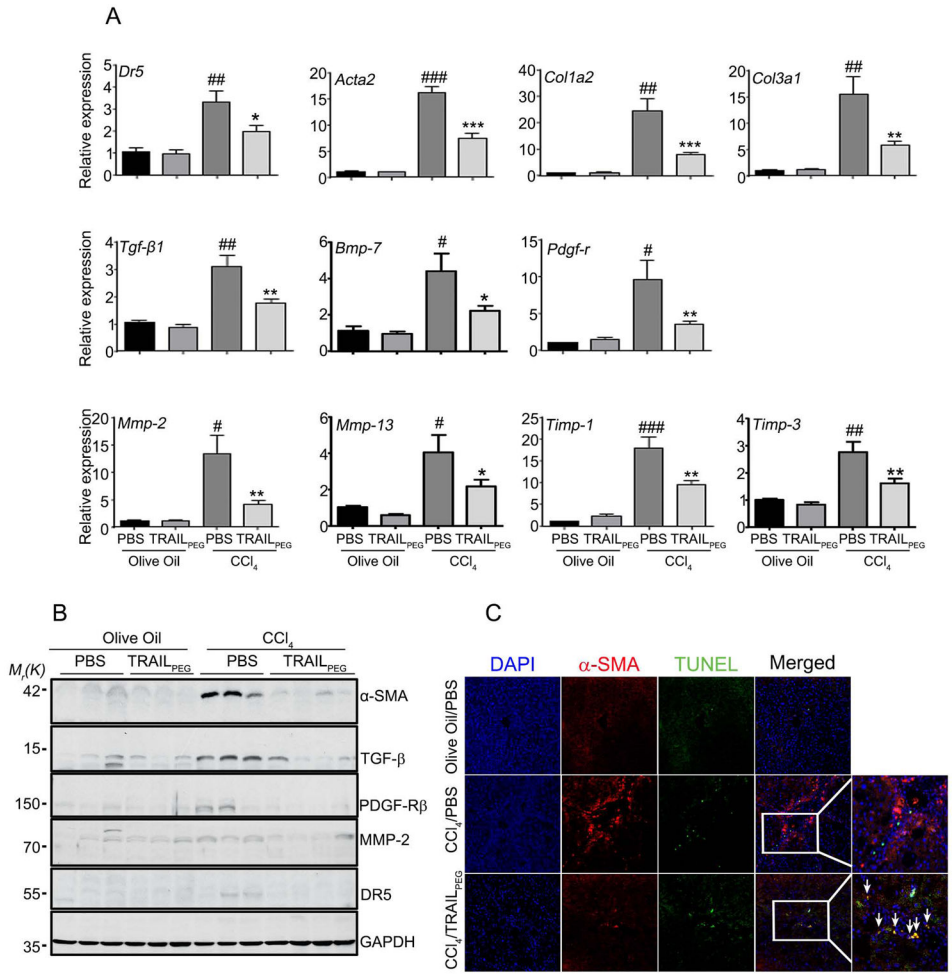

Figure 3. Intravenously injected TRAIL$_{PEG}$ effectively targets aHSCs and simultaneously downregulates multiple fibrogenic components during liver fibrogenesis

(A) Downregulated gene expression profiles of TRAIL receptor (DR5), HSC activation and fibrogenic markers from TRAIL$_{PEG}$ treated fibrotic livers (n = 4–10). (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 100x. Arrows indicate TRAIL$_{PEG}$-induced apoptosis in aHSCs. Arrows indicate overlap of TUNEL and α-SMA staining. Additional markers in the sera of rats are shown in Supplementary Figure 3. Data expressed as mean ± s.e.m. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. olive oil + PBS groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CCl_4 + PBS groups.

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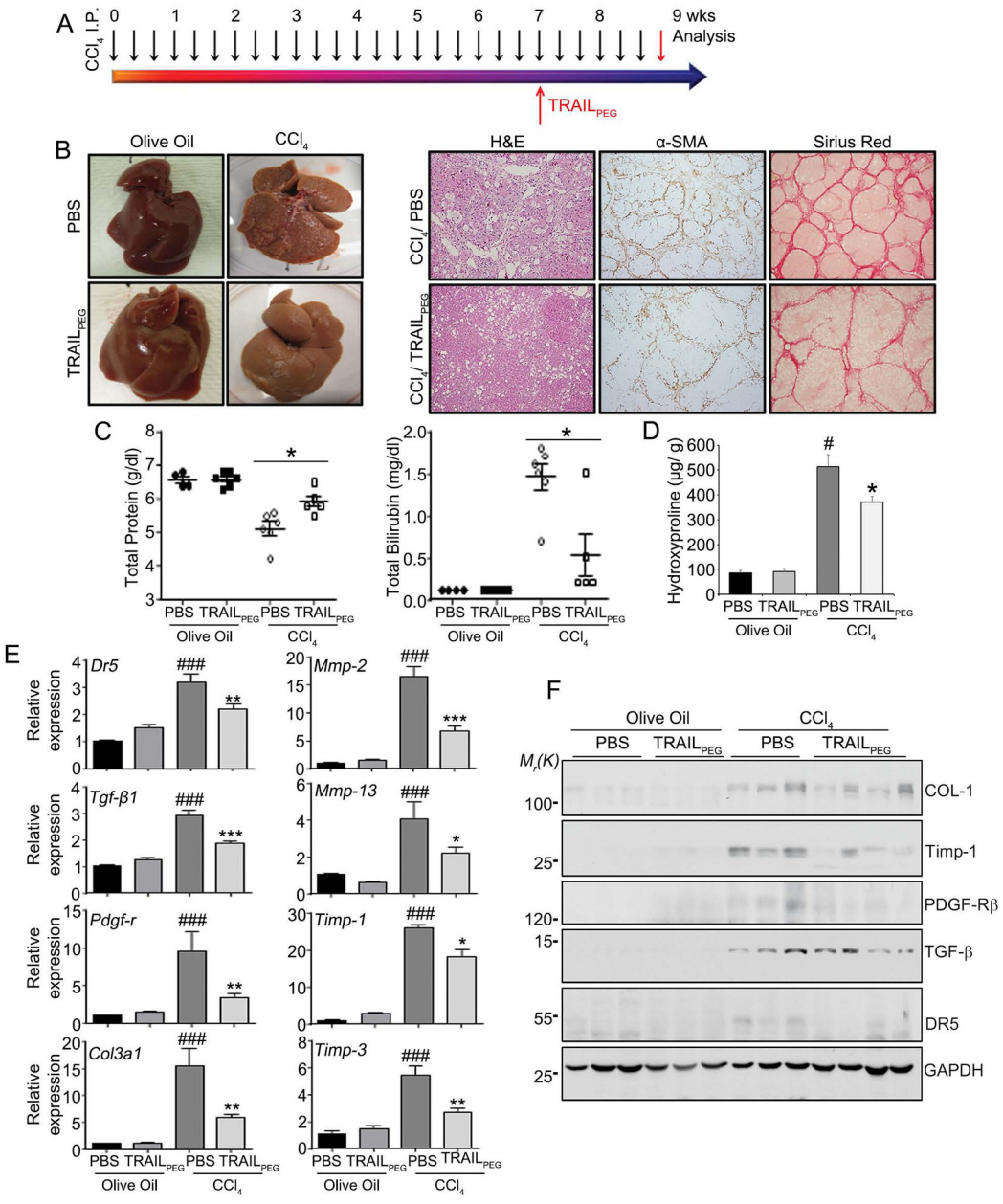

Figure 4. Intravenously injected $TRAIL_{PEG}$ ameliorates CCl_4-induced liver cirrhosis in rats

(A) $TRAIL_{PEG}$ (4 mg/kg) was intravenously treated daily for two 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 photos of normal and cirrhotic livers from rats (left) and representative photomicrographs of liver sections stained with H&E and immunohistochemistry of activated HSC marker (α-SMA) and collagen deposition (Sirius Red) treated with or without $TRAIL_{PEG}$, according to timeline in (A), at day 65. Original magnification 40x. (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

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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). (F) Western blot analyses of TRAIL receptor (DR5) and fibrogenic markers. Control (Ctrl) is PBS-treated normal (olive oil-treated) liver samples. Additional markers in the sera of rats and gene expression profiles are shown in Supplementary Figure 6B. Data expressed as mean ± s.e.m. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. olive oil + PBS groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CCl_4 + PBS groups.

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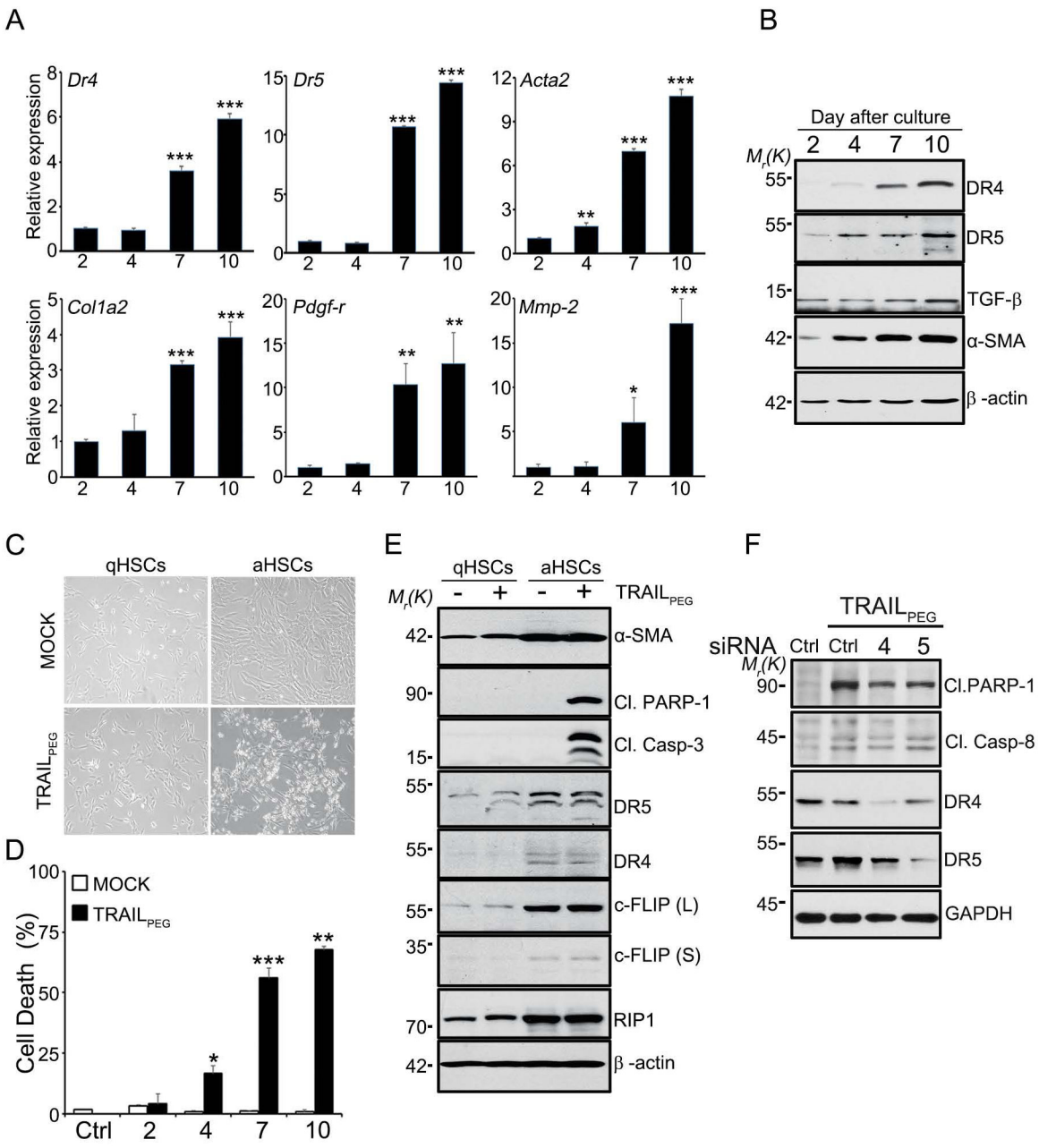

Figure 5. HSCs upregulate fibrogenic components and death receptors during activation and become sensitive to TRAIL$_{PEG}$-induced apoptosis

Human primary HSCs were culture-activated for various time points (2–10 days) and treated with TRAIL$_{PEG}$ or vehicle followed by quantitative real time PCR (qPCR) or western blot analyses. (A) qPCR 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 anti-apoptotic and pro-apoptotic 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 a activation bio-marker for HSCs. (C) Representative photos of qHSCs (day 2) and aHSCs (day 7) treated with TRAIL$_{PEG}$ (1 μg/mL) for 3 h. (D) Quantified

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$TRAIL_{PEG}$-induced cell death during HSC activation (day 2–10) analyzed by MTT assay. (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 h. (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 h. Data expressed as mean ± s.d. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. qHSC (day 2) (A) or MOCK (D).

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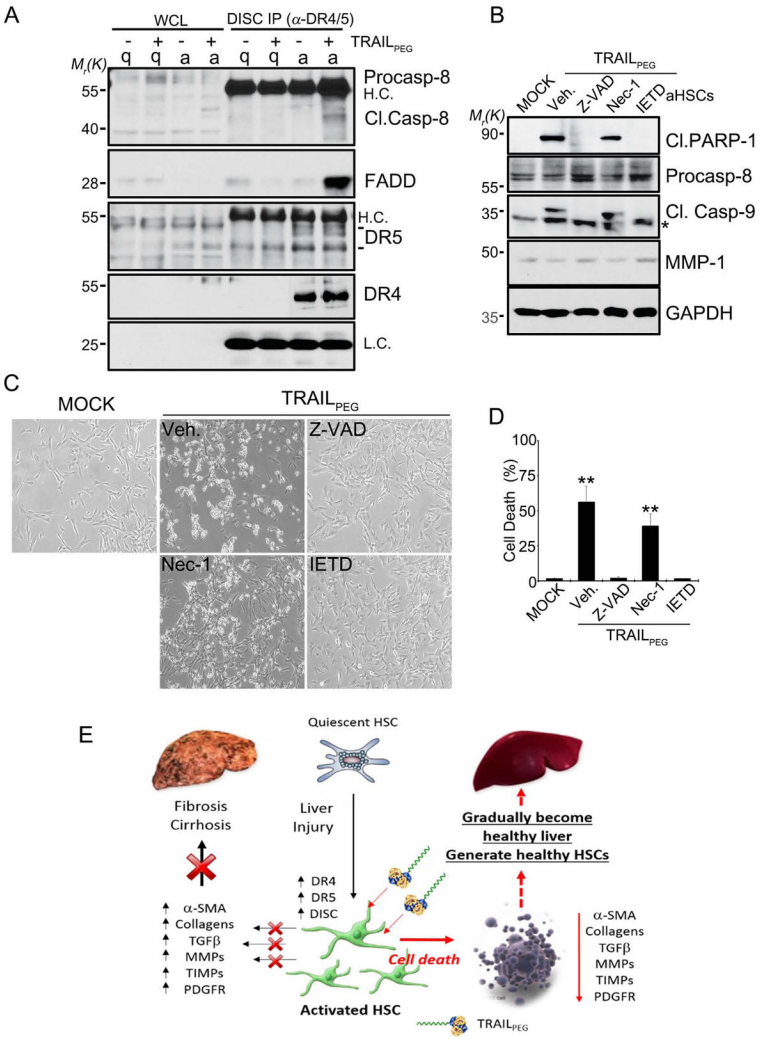

Figure 6. TRAIL$_{PEG}$ induces apoptosis in aHSCs, but not quiescent HSCs (qHSCs), through death-inducing signaling complex (DISC) and caspase-dependent pathway

(A) DISC immunoprecipitation (IP) studies. Western blot analysis of qHSCs (q, 2 days) and aHSCs (a, 7 days) treated with TRAIL$_{PEG}$ (2 μg/mL, 60 min) 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 co-treated 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 h. (C) Representative photos of aHSCs co-treated 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 h. (D) Quantified cell death analyses by MTT assay. Data expressed as mean ± s.d. *$P < 0.05$, **$P < 0.01$ vs. MOCK (D). (E) Summarized effect of TRAIL$_{PEG}$ on HSC activation and liver fibrogenesis.