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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*  
2016

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Arabpour, M. (2016). *Addressing liver fibrosis by TRAIL targeted to hepatic stellate cells*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

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# Chapter 4

## **Elimination of activated hepatic stellate cells by receptor-specific TRAIL**

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## Abstract

**Background** The activated hepatic stellate cell (HSC) is the major natural target in resolving liver fibrosis. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a molecule that may contribute to the apoptotic removal of activated HSC through binding to its dedicated receptors. In the present study, we investigated the potential application of recombinant receptor-specific TRAIL proteins in the efficient elimination of activated HSCs.

**Methods** Human LX-2 cell line or rat HSCs were activated by progressive culture on plastic surface. TRAIL receptors expression on the surface of cells were profiled for each specific receptor in activated HSCs. Activated HSCs were treated by receptor-specific TRAIL variants *in vitro* to evaluate its effect on the cell viability and extracellular matrix production.

**Results** Our finding revealed differential contributions of TRAIL receptors among HSCs populations. Activated HSCs express more TRAIL receptors. Also, HSCs dominantly express DR5 receptors; whereas the side population of HSCs mostly expresses DcR2. *In vitro* treatment of activated HSCs with DR5-specific or wild type TRAIL variants induced a significant reduction in viability and extracellular matrix production. Decrease in viability was associated with treatment of cells only in the presence of high concentration of DR4-specific TRAIL. A decrease in extracellular matrix production was found to correlate with increasing concentration of all TRAIL variants. A pronounced reduction (up to 100%) in collagen production was observed after treatment with DR5-specific TRAIL.

**Conclusion** our findings provide evidence that more than one receptor system is involved in the recognition and signal transduction of TRAIL into activated HSCs. Evidence is presented for the successful application of the DR5 receptor-specific TRAIL variant in the targeted elimination of activated HSCs via interference with collagen production and simultaneous induction of apoptosis via activation of the caspase pathway. DR5 receptor-specific TRAIL may thus represent a new therapeutic compound for the treatment of liver fibrosis.

## **Introduction**

Following chronic injury, the liver develops to a pathologic state regarded as fibrosis. The key factor in the liver fibrosis process is a cell type called the HSC. Quiescent HSC are dedicated to retinoid storage, yet through an activation process due to injury, they proliferate and transform to a myofibroblastic phenotype. In this form activated HSCs start secretion of extracellular matrix proteins mainly collagen I and III that accumulate over time and impair the functional structure of the liver [1]. During activation, HSC become more susceptible to apoptotic cell death by apoptotic factors, including Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL). TNF $\alpha$ , FasL and TRAIL are among the most studied factors in the tumor necrosis factor family members that employed by a number of immune cells, specially natural killer cells(NK) and Cytotoxic Lymphocyte (CTL), to induce controlled apoptosis in tumor cells or infected cells [2]. Whereas TRAIL, CD59L and TNF $\alpha$  may have a proliferative effect on HSCs in certain conditions[3–

5] and could cause massive hemorrhagic necrosis of various tissues, including the liver[6]. Thus TRAIL is the most promising apoptotic ligand to eliminate activated HSCs. There are two death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) that are responsible for induction of apoptosis upon binding to TRAIL, yet TRAIL also can bind to the anti-apoptotic decoy receptors DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (OPG). The application of TRAIL agonists has been suggested as a potential strategy to eliminate activated HSCs[7,8]. On the other hand, the ubiquitous expression of TRAIL receptors and the complex role of apoptosis in inducing inflammation make it difficult to explore TRAIL ligands for the treatment of liver fibrosis[9,10]. TRAIL receptor-specific agonistic molecules have been introduced both in the form of TRAIL variants or monoclonal antibodies against specific TRAIL receptors[11,12]. The dynamics of wild type (wt) and mutant TRAIL interactions with TRAIL death receptors have been well characterized[13]. Receptor-specific agonists may reduce the decoy receptor-mediated antagonism[14], hence by using receptor-specific TRAIL variants the therapeutic dose is expected to be lower[15,16]. The application of receptor-specific agonists for DR5 drastically reduced hepatotoxicity [17] that is associated with wild type human TRAIL [18–21]. DR5 specific TRAIL lower toxicity could be attributed to negligible amount of DR5 on the surface of healthy hepatic cells in comparison to DR4 [20,22]. In this study, we investigate the potential application of two receptor-specific TRAIL variants for targeted elimination of activated HSCs.

## **Material and methods**

### **Cell lines and culture**

The LX-2 immortalized human HSC line was kindly provided by Prof. Scott Friedman (Mount Sinai Hospital, New York) and was cultured in Dulbecco's minimum essential medium (DMEM; Gibco, Glutamax) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 g/mL streptomycin, 50 g/mL gentamicin, and 100 nmol/L insulin. The HepG2 human hepatoma cell line (ATCC#HB-8065), and HEK-293 human embryonic kidney cell line (ATCC# CRL-1573) were cultured in DMEM (Gibco,) containing 10% fetal bovine serum. Cells were cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C.

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### **Primary hepatic cells**

**Animals** Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, the Netherlands). They were housed under standard laboratory conditions and had free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

### **Isolation and culture of rat hepatocytes and HSCs**

Primary rat hepatocytes [23] and HSCs [24] were isolated and cultured as described previously. Hepatocyte viability and purity were always more than 90% as judged by Trypan blue exclusion. Cells were cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C.

### **Antibodies and TRAIL variants**

The following antibodies (Ab) were used: Anti-TRAIL monoclonal Ab anti-Human CD253 (TRAIL) (eBioscience Affymetrics Co); Mouse anti- human EGFR IgG Ab; Mouse anti- human  $\alpha$  Smooth Muscle Actin ( $\alpha$ -SMA) IgG Ab; Goat anti- human collagen I IgG Ab; Anti- $\beta$  actin mouse IgG (Sigma). Receptor-specific TRAIL proteins DHER and 4C7 were produced and purified from prokaryotic expression systems as previously described[19,21]. For the apoptosis assay Annexin V-FITC (IQ products, IQP-120F) and Mouse IgG2b-FITC (IQ products, IQP-193F) was used. DR4-specific, DR5-specific and wild type TRAIL antibodies were used as previously described[16].

### ***In vitro* evaluation of receptor-specific TRAILs for inducing apoptosis in HSCs**

To activate the LX-2 cells or primary rat HSCs, cells (1000 cells /well) were seeded and incubated in 96-well, flat-bottomed uncoated plastic plates in DMEM, Glutamax (Invitrogen) media with 10% FBS for up to 7 days as previously described[25]. HSCs were exposed for 48 hours to various concentrations of WT TRAIL or receptor-specific TRAIL variants DHER or 4C7, diluted in DMEM medium. Primary hepatocytes were seeded at a concentration of (8000 cells/well) in 96-well, flat-bottomed uncoated plastic plates overnight before treatment with receptor-specific TRAILs for 48 hours. The effect of different proteins and agents on cell viability were assessed using MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate (PMS)) according to the manufacturers protocol (Sigma) in triplicate. Cell only controls received DMEM 1% FBS.

### **Immunohistochemistry and Western blotting**

For SDS-PAGE and Western blotting, LX-2 cells were cultured to 50% confluency. At the indicated time points TRAIL proteins were added at concentrations ranging from 10 to 100 ng/ml in DMEM. For control cells, DMEM was added. 48 hours later the supernatants were removed and the cells were washed with PBS. The cell pellets were separated on 12.5 % SDS- polyacrylamide gels as previously described[26]. For Western Blot analysis with Abs against collagen I,  $\alpha$ -SMA and  $\beta$ -Actin, gels were blotted on blotting membrane as previously described [26] , followed by incubation for 2 hours at room temperature with either an Ab against human collagen I (1:1000),  $\alpha$ -SMA (1:1000) or  $\beta$ -Actin (1:5000) in PBS with 1% BSA and 0.05% Tween-20. After washing, the membrane was incubated for one hour with the second Ab (horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG,; Dako, Denmark) for  $\beta$ -Actin and  $\alpha$ -SMA or mouse anti-Goat IgG-HRP (Dako, Denmark ) in the case of collagen I, all diluted 1:1000 in the buffer mentioned above. The membrane was then washed four times for 15 minutes in the washing solution as above. The blot was developed using an AEC staining solution system (Sigma, USA) according to the manufacturer's instructions.

For Immunohistochemistry of cultured activated LX-2 cells, first LX-2 cells were cultured on uncoated plastic in 96-well plates with 1% FCS in DMEM for 7 days, and incubated with different concentrations of TRAIL. After 24 hours the medium was removed and the cells were washed 3 times with PBS, fixed with absolute methanol (Sigma) at -20°C for 20 minutes, air-dried for 30 minutes and re-hydrated with PBS for 5 minutes. Next, the cells were incubated with either anti-collagen I



(1:75), anti- $\alpha$ -SMA (1:600) in PBS or 1:10,000 Hoechst 33342. Finally, the cells were washed with PBS 3 times and incubated with the second Ab (Rabbit anti-mouse IgG-HRP; Dako, Denmark) for  $\alpha$ -SMA and (mouse-anti Goat IgG-HRP; Dako, Denmark) for Collagen I all diluted 1:100 in PBS. The integrated density of intracellular insoluble collagen or  $\alpha$ -SMA was then calculated through analysis of five separate pictures from triplicate experiment for each treatment. All acquisition parameters were kept constant for samples and respective controls. Images were imported into Image J 1.48 software. For each wavelength back ground fluorescence has been subtracted from the selected region of interest. The integrated cell density was then calculated and normalized for the total number of cells for all pictures.

#### **Profiling TRAIL receptor expression on HSCs**

Flow cytometer analysis of TRAIL receptors was performed as follows. HSCs were detached using 0.05% Trypsin-EDTA in PBS, washed with ice-cold PBS, and diluted to a concentration of  $2 \times 10^6$  cells/mL using cold PBS. Aliquots of 100  $\mu$ L ( $2 \times 10^5$  cells) were centrifuged at 14,000 rpm for 5 min at 4°C, the supernatant was discarded, and the cells were separately suspended in 100  $\mu$ L of rabbit anti-DR4 (10  $\mu$ g/mL), rabbit anti-DR5 (10  $\mu$ g/mL), rabbit anti-DcR1 (10  $\mu$ g/mL) or rabbit anti-DcR2 (10  $\mu$ g/mL) antibodies for TRAIL DR5 and incubated for 45 min at 4°C. The cells were then washed twice with PBS and were incubated for an additional 45 min with 1:100 diluted anti-rabbit PE-conjugated secondary antibody under cold and dark conditions. After two final washings, cells were suspended in 300  $\mu$ L PBS (containing 1%BSA). Unlabeled cells and cells labeled with secondary antibody

alone served as negative controls. The mean values of fluorescence intensity of 10,000 cells were determined by flow cytometry analysis (Calibur 1).

For analysis of TRAIL receptor distribution in activated HSC side populations (SP), LX-2 cells cultured on plastic plates for 7 days in DMEM containing 10% FBS were detached by trypsinization for 3 min. The number of viable cells was estimated by Trypan blue staining. Cells were collected by centrifugation and washed with 5 mL of phosphate-buffered saline (PBS). To detect the SP, LX-2 cells ( $1 \times 10^6$ ) were incubated with Hoechst 33342 (5 mg/mL) in DMEM/10% FBS for 90 min at 37°C with vortexing every 15 min. Cells were then stained with specific TRAIL receptor antibodies as explained above. As previously described [27] to discriminate between SP and non-SP cells, cells were incubated with the Hoechst dye in the presence or absence of verapamil (50 mM). At the end of the incubation period, 1 mg/mL propidium iodide (PI; Sigma) was added to identify dead cells before analysis by flow cytometry for Blue 450nm (blue Hoechst) and 675 (red Hoechst) laser using the BD LSR II Flow Cytometer [27]. Gated SP cells were analyzed for expression of different TRAIL receptors. All FACS data analyzed with FlowJo VX software.

#### **RNA Isolation and Real-Time RT-PCR Analysis**

Total RNA from cultured LX-2 cells was isolated using the SV total RNA isolation system (Promega Z3100) (all tests carried out in triplicate). The amount of RNA was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and analyzed qualitatively by gel electrophoresis. Subsequently, synthesis of first-strand cDNA from total RNA was performed with Revers Transcription system (Promega A3500) in a volume of 20  $\mu$ l containing 250 ng of

oligo dT (Promega, Madison, WI). The obtained cDNA was diluted with Millipore water (Millipore Corporation, Billerica, MA) to a concentration of 10 ng/ $\mu$ l, and 1  $\mu$ l was applied for each PCR reaction. The  $\alpha$ -SMA primer was ordered as Assays-by-Design (Applied Biosystems, Foster City, CA) (4331348/assay name ACT-R-ACT2). GAPDH was used as a housekeeping gene (Applied Biosystems). DR5- and DR4-specific receptors primers were used as previously described[25]. The PCR reaction was carried out in TaqMan PCR Master Mix (Applied Biosystems) with a final concentration of 200 nM GAPDH primers and 250 nM for primers of the other genes studied. The amplification reaction was performed in an ABI PRISM 7900HT sequence detector (Applied Biosystems) with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. For each sample, the real-time PCR reaction was performed in triplicate, and the averages of the obtained threshold cycle ( $C_t$ ) values were processed for further calculations according to the comparative  $C_t$  method described in the ABI manual (<http://www.appliedbiosystems.com>). In brief, gene expression levels were normalized to the expression of the housekeeping gene GAPDH, giving the  $\Delta C_t$  value. The average value of  $\Delta C_t$  obtained from day 0 culture LX-2 was subtracted from the average of the  $\Delta C_t$  value of each sample, yielding the  $\Delta\Delta C_t$  value. Finally, the gene expression level was calculated as  $2^{-\Delta\Delta C_t}$ , giving the final value that is normalized to the housekeeping gene and relative to the control sample values of the studied  $\alpha$ -SMA gene.

### **Total soluble collagen assay**

The Sircol assay (Biocolor, UK) was used for measuring soluble collagen in culture supernatants. One ml of Sircol dye reagent and 150  $\mu$ l of sample were mixed and incubated for 30 min at room temperature. Collagen–dye complexes were centrifuged for 10 min at 14,000rpm, washed with 1 ml of ethanol and dissolved in 1 ml of 0.5 M NaOH. The absorbance was measured at 540 nm. The assay was performed in triplicate, and the mean of data was calculated for each sample.

### **Caspase and Apoptosis assay**

Caspase 3/7 activities were assayed using the Apo-ONE™ Homogeneous Caspase-3/7 Assay kit (Promega). Briefly,  $1 \times 10^4$  HSCs treated with 100 ng/ml of the different TRAIL variants or DMEM medium (control). Cells were transferred into a 96-well plate and 100  $\mu$ l of Homogeneous Caspase-3/7 Reagent was added. The plate was covered with a plate sealer. After incubation for 2 hours, fluorescence of each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The measurements are reported in relative fluorescence units (RFU).

For evaluation of apoptosis, LX-2 cells were treated with TRAIL variants as described above and harvested at the indicated time points. Briefly, cells were washed with calcium buffer (0.15 M NaCl, 11.7 mM HEPES and 23 mM  $\text{CaCl}_2$ ) buffer and re-suspended in 1/10 dilution of anti-Annexin V in 60  $\mu$ L of calcium buffer. Cells were incubated for 30 minutes at 4°C, washed in calcium buffer and re-suspended in calcium buffer supplemented with 0.5  $\mu$ g/ml propidium iodide. Finally, fluorescence-activated cell sorting (FACS) was performed using the BD LSR II

Flow Cytometer. Gated cells were analyzed for apoptotic signaling of Annexin V. All FACS data were analyzed with FlowJo VX software.

### **Statistical analysis**

All experiments were performed in triplicate and averages were used to calculate for significance between different treatments. The significance of the difference in TRAIL receptors expression was analyzed by the Student's *t* test on three separate experiments. *P* values <0.05 were considered to be significant. Comparisons between different primary cells and or cell lines treated with TRAIL variants were calculated by using a non-parametric one-ways ANOVA with Sidak's multiple comparison tests with a single pooled variance (\**P* < 0.05) using GraphPad Prism version 5.0 for Windows (GrahPad Software).

## **Results**

### **TRAIL receptors expression increases during progressive activation of LX-2 cells in cell culture**

LX-2 cells were activated by culturing the cells for 9 days on polyethylene plastic plates This model of progressive activation in culture has been established for LX-2 cells for the *in vitro* study of liver fibrosis[25,26]. LX-2 cells were harvested and screened for signs of activation via mRNA expression of  $\alpha$ -SMA, an indicative marker of activation, during different time points. An increase of five-fold in mRNA and protein expression of  $\alpha$ -SMA was associated with progressive activation (Fig. 1A). This model was further used to evaluate the effect of different TRAIL variants

on activated HSCs. We assessed the expression of the different TRAIL receptors using both real-time PCR and FACS analysis. Real time analysis showed that there was an increased DR4 and DR5 receptor mRNA expression in accordance with the observed activation profile for  $\alpha$ -SMA expression in activated HSCs. The increase in mRNA expression was 100-times more for the DR4 receptor compared to the DR5 receptor (Fig. 1B). Flow cytometry analysis showed that there was a 100% and 10% shift in mean fluorescent intensity of DR4 and DR5, respectively. However, the level of DR5 expression in comparison to DR4 was 10-fold higher, which could indicate that DR5 may play a more important role in TRAIL-mediated apoptosis of HSCs (Fig. 1C) (Supplementary Fig. 1). The expression of both DcR1 and DcR2 seem to be absent on *LX2* cells and only detectable at low levels in activated *LX2* cells.

#### **TRAIL receptor 5 is expressed in major HSC population while TRAIL decoy receptor 2 is expressed in SP HSC population**

Side populations (SP) have already been identified in bone marrow, skeletal muscles and liver cells. These cells are characterized as progenitor cells that can actively efflux the red Hoechst 33342 dye. The HSC SP has been characterized as a part of the liver SP cell type that can regenerate and transdifferentiate[28–30] . Sustainability of this cell population has been shown through long-term culture *in vitro*. Interestingly, TGF- $\beta$  signaling reduces HSCs SP and renders this population susceptible to the fibrotic process where TGF- $\beta$  plays a major role [17]. Due to the regenerative role of the HSC SP a better understanding of the TRAIL receptors contribution to this population could be helpful in identifying a suitable TRAIL

ligand for the treatment of fibrosis. Using the flow cytometry analysis of blue and red Hoechst dyes, we found that the HSC SP consists 1.6 % of the activated LX-2 cells (Fig. 2). The expression of TRAIL receptors on HSC SP was determined. Around 95% of HSCs expressed DR5 on their surface (Fig. 1), whereas negligible amount of SP HSCs expressed DR5 receptors on their surface. Also, around 33% and 10% of SP cells expressed DCR2 and DCR1 respectively. DCRs expression is negligible in the rest of the population (Fig. 2). These result indicated that the HSC population maintained high level of DR5 receptor expression in culture; whereas SP HSCs maintained low levels of TRAIL death receptors and higher level of TRAIL decoy receptors.

**DR5-specific TRAIL decreases Extra Cellular Matrix (ECM) production by activated HSC**

One of the characteristic of activated HSCs is the increased expression of fibrotic markers such as  $\alpha$ SMA and collagen I. It has been previously been shown that TRAIL and TRAIL derivatives could reduce the expression of ECM produced by activated HSCs[26,31]. To evaluate the potential effect of receptor-specific TRAIL on collagen I expression, LX-2 cells were cultured on a plastic surface for 7 days and different amounts of receptor-specific or wt TRAIL were added to cell cultures. After 48 h, cells were analyzed for collagen production using the Sircol assay and western blotting. Both DR5 and wt TRAIL caused a substantial decrease (80%) in collagen production, even at very low dose (10 ng/ml), whereas DR4-specific TRAIL was only capable of a similar effect at a high dose (Fig. 3A). It has been reported that the expression of collagen correlates closely with the expression of collagen-specific molecular

chaperone Hsp47 in activated HSCs. Therefore, we next investigated the expression of HSP47 in LX-2 cells treated with increasing amounts of TRAIL variants. The results suggest that a decrease in collagen production was associated with down regulation of HSP47 in TRAIL-treated cells. Furthermore, DR5 receptor-specific TRAIL showed a greater capability in reducing HSP47 expression at lower concentration (Fig. 3B, C). However, the reduction of collagen was significantly higher in DR5 receptor-specific TRAIL compared to the DR4 receptor-specific TRAIL. Finally, staining of TRAIL treated LX-2 cells with anti-collagen I and anti  $\alpha$ -SMA revealed increase in intracellular insoluble collagen is associated with decrease in  $\alpha$ -SMA. Moreover the aggregation of insoluble collagen and reduction in the expression of  $\alpha$ -SMA were more pronounced in cells treated with DR5-specific TRAIL, thus indicating with DR5 specific TRAIL seems to interfere more efficiently with collagen folding mechanism. (Fig. 4).

#### **Different receptor-specific TRAILs have different potential to eliminate activated HSCs**

To evaluate the potential cytotoxic effect of receptor-specific TRAIL, different amounts from each receptor-specific or wt TRAIL protein were added to activated or quiescent LX-2 HSCs and the viability of cells was determined 72 hours later by the MTS assay (Figure 5). Activated LX-2 cells showed a substantial decrease in viability by increasing doses of TRAIL. A comparison between the different TRAIL variants showed that DR5-specific TRAIL was more potent in reducing viability (60 %), whereas treatment with DR4-specific TRAIL did not affect the viability of activated HSC, only in the presence of high concentrations of TRAIL (Fig. 5B). Treatment of quiescent LX-2 with different types of TRAIL, on the other hand, did



not significantly affect the viability even at the highest concentrations (1 µg/ml) (Fig. 5A). Finally, to examine the specificity of the different TRAIL variants to HSCs, the hepatic parenchymal cancer cell line HepG2 was treated with increasing concentrations of DR4, DR5 and wt TRAIL. Whereas no detectable difference in viability of HepG2 cells was observed after application of wt TRAIL, a decrease in viability was observed with the DR4 or the DR5 specific TRAIL proteins (Fig. 5C). These findings confirm the selectivity of the wt TRAIL protein towards activated HSC.

The inhibition of the viability in primary activated rat HSC was lower than in LX-2 cells and was only found at high doses of DR5-specific TRAIL (1 µg/ml), which showed a 20% reduction in viability (Fig. 5E). No significant reduction in viability was observed after treatment with the different TRAIL variants of quiescent rat HSCs (Fig. 5D) or rat hepatocytes (Fig. 5F).

We next evaluated the contribution of the different TRAIL receptor variants with respect to induce apoptosis using the caspase 3/7 activity assay. The measurement of caspase activity in HSCs treated with TRAIL showed a higher caspase activity for DR5- and WT-treated cells versus DR4-treated LX-2 cells. Data obtained were normalized to the untreated cell line (Fig. 6A).

To further investigate the apoptosis-inducing activities of the different TRAIL variants, LX-2 cells were incubated with PBS or the TRAIL variants proteins for 48 hours. Consistent with the cell viability results, only wt and DR5-specific TRAIL induced robust apoptosis of LX-2 cells, whereas DR4-specific TRAIL was less effective (Fig. 6B) as measured by Annexin V FACS analysis.

## Discussion

Persistent liver injury lead to enhanced ECM production and accumulation, a condition referred to as liver fibrosis. HSCs have a central role in this process, it has been shown that activated HSCs significantly amplify the hepatic response to liver injury, hence depleting fibrotic livers from activated HSCs may ameliorate the fibrotic condition [32]. TRAIL-induced apoptosis was successfully used to eliminate activated HSC [25]. In order to increase the specificity and to be able to lower the effective dose of TRAIL we evaluated the different properties of receptor-specific TRAILs as a selective treatment for the elimination of activated HSCs to improve the remedy from liver fibrosis. To uncover the dynamics of TRAIL receptor expression during time-dependent HSC activation, the expression pattern of different TRAIL receptors on the surface of quiescent and activated HSCs was evaluated. Our findings cast light on the differential contribution of TRAIL receptors in HSCs. An increase in the level of TRAIL DR5 and DR4 receptors was observed both in mRNA and protein expression upon activation of HSCs (Fig. 1). Despite the increase in TRAIL-DR4 receptor expression during activation, the net expression remained significantly lower than that of the TRAIL-DR5 receptor. An increase in the expression of TRAIL receptors was concomitant with an increase in expression of TRAIL decoy receptors, especially DcR2. As part of our study we demonstrated that the DR5 receptor was the single most dominant receptor on HSCs in comparison with the HSC side population, whereas decoy receptor DcR2, was predominantly expressed on the surface of HSC side population. This finding is particularly important since It has been proven that HSCs may directly undergo

mesenchymal-to-epithelial transition to transdifferentiate into liver progenitors [25] or indirectly augment regeneration of hepatic cells by its produced cytokines [26][27]. The exact mechanisms by which HSCs may help mediate liver regeneration and the relative importance of different subtypes of HSCs remain to be determined. However, crucial regenerative role of the SP in multiple organs imply the possibility for this population to have similar role in liver regeneration. Thus, next to the higher expression of TRAIL receptors on activated HSCs the selectivity of TRAIL variants could be partially explained by the higher expression of the decoy receptors in SP HSCs than in rest of population. Our findings therefore, show the protective role of TRAIL decoy receptor 2 in HSCs SP.

To further evaluate the application of different receptor-specific TRAIL agonists in eliminating activated HSCs we explored the functional capacity of receptor-specific TRAIL variants [15,16][36] in inducing apoptosis and reducing ECM production in HSCs. ECM-producing cells, such as HSC, are highly responsive to a series of factors such as EGF, PDGF and TGF- $\beta$  that are released from damaged liver parenchyma during the fibrotic process. Affected HSCs start producing excessive amount of ECM. The production of ECM, especially Collagen I, could in return accelerate HSC proliferation and fibrosis [37,38]. Addressing this phenomenon, we evaluated the capability of different TRAIL variants in blocking ECM production by HSCs. All TRAIL variants suppressed the production of soluble collagen by activated HSCs with DR5 receptor-specific and wt TRAIL being the most potent molecules. This observation was accompanied by down regulation of the pro-collagen specific molecular chaperone Hsp47 and aggregation of insoluble collagen

in TRAIL-treated cells. These findings cast light on the supporting mechanism of receptor-specific TRAIL mediated collagen regulation through interfering with the natural folding and secretion process of pre-collagen in activated HSCs. A direct relation has already been established between fibrogenesis and the number of  $\alpha$ -SMA positive cells [39]. Since the increase in accumulation of insoluble collagens in our study was associated with a reduced number of  $\alpha$ -SMA positive cells, it may be speculated that the decreased expression of ECM proteins is caused by the reduction in HSC activation and proliferation. The exact mechanism for this phenomenon yet to be defined, but it has been shown that TRAIL could inhibit cellular growth in the absence of cell death induction due to cyclin dependent cell cycle arrest in S-G2/M phase[40–42]. Hence, inhibition of HSCs activation and proliferation by TRAIL could directly reduce secretion of ECM including collagen I. An alternative mechanism for reduced activation of TRAIL affected HSCs could be attributed to role of pericellular collagen on HSCs proliferation. There is accumulating evidence that HSC biology is tightly regulated by their peripheral ECM environment. It has been shown collagen I, the major component of fibrosis, enhances the proliferation of HSC *in vitro* via different mechanisms i.e. interacting with type 2 discoidin domain receptor [43,44]. Thus, TRAIL property for *in vitro* eliminating ECM accumulation through interfering with pro-collagen folding and secretion could eliminate HSCs proliferation and promote reversion of the fibrotic phenotype of activated HSCs[45].

Our findings revealed a substantial decrease in LX-2 HSC viability achieved through exposure to DR5-specific and wt TRAIL, whereas DR4-specific TRAIL showed

only a marginal effect on LX-2 cell viability. This finding is in concordance with findings regarding the functional role of different TRAIL variants in inducing apoptosis as measured by increase in caspase 3/7 and Annexin V [25]. Selectivity of TRAIL variants is an important issue since apoptosis in itself could have a dual function in ameliorating or deteriorating fibrosis progression. Here, we showed that the effects of receptor specific TRAIL variants are limited to the HSCs rather than the hepatocytes. The observed sensitivity of HepG2 cells towards receptor-specific TRAIL could be partially explained through its over expression of pro-apoptotic TRAIL receptors and DcR2 in hepatocellular carcinoma cell line [46]. Since fibrosis is considered rather a long term and persistent problem, a durable and efficient approach need to be pursued. Our current strategy to dissect the functional properties of different TRAIL variants is particularly beneficial since TRAIL shows a very short half-life (6 hours) *in vivo* and thus needs high dosages and frequencies of administration for clinical benefit. We speculate that an advantage of treatment with receptor-specific TRAIL variants is the specific targeting of activated HSCs and lowering required dosage for having the same effect. Moreover, given that TRAIL variants carry both separate functions for simultaneously inducing apoptosis through TRAIL receptors and reducing ECM production via blocking relevant signaling pathways in HSCs, it appears to be an ideal combination for eliminating fibrotic process. Hence, a loss of viability in TRAIL treated activated HSCs could be followed by a lower proliferation and reversion to quiescent phenotype of remaining activated HSC. This is particularly important for sustaining the effect of TRAIL. Furthermore, TRAIL variants may be applied as genetic therapy [47] resulting in

highly effective long-term *in vivo* elimination of activated HSCs and reduction of ECM production. In this regard, our study is the first to show a significant anti-fibrotic effect of different receptor-specific TRAIL variants, which warrants further *in vivo* studies.

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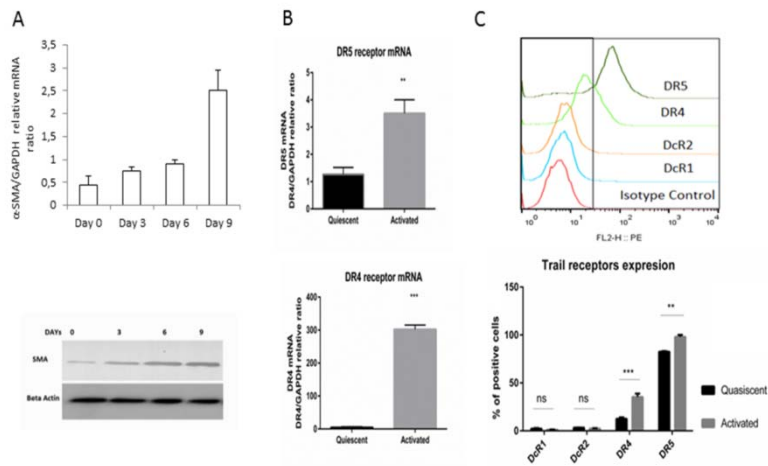
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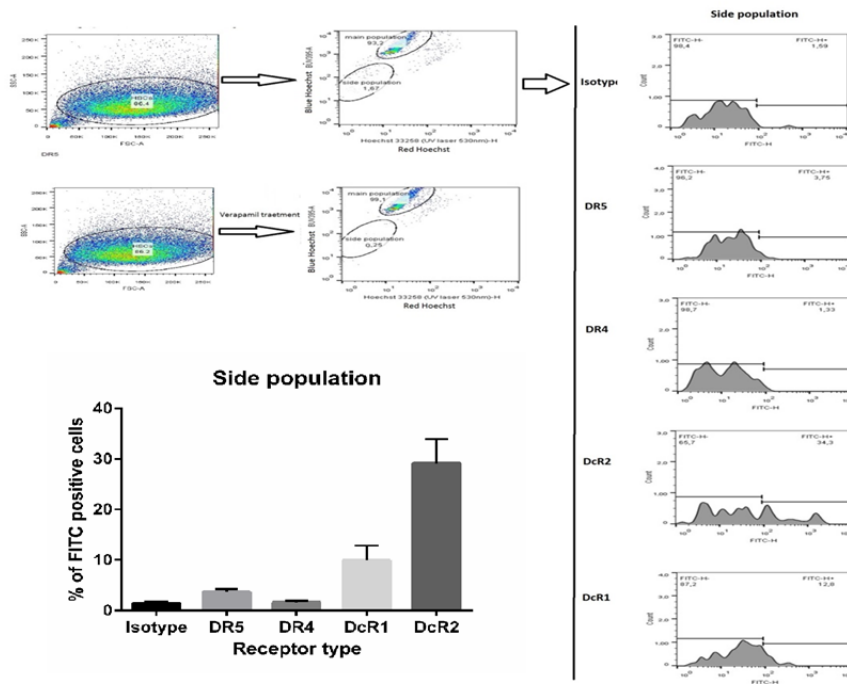
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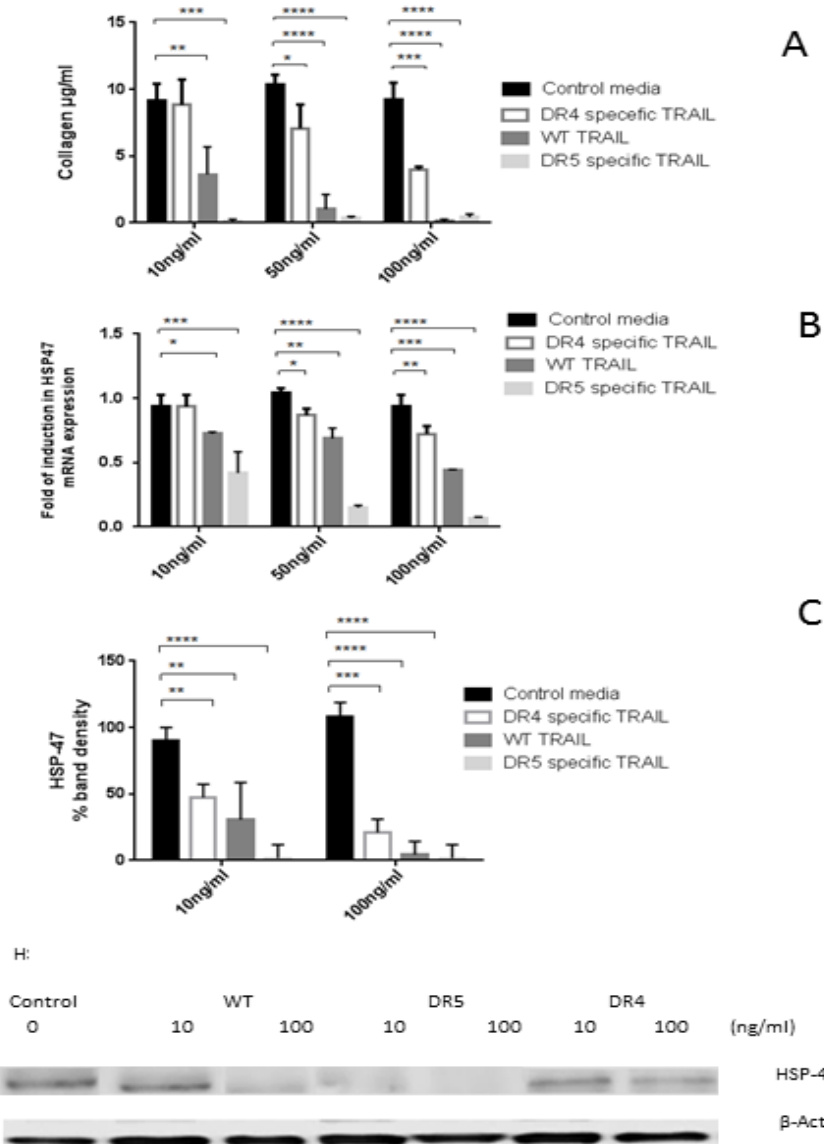
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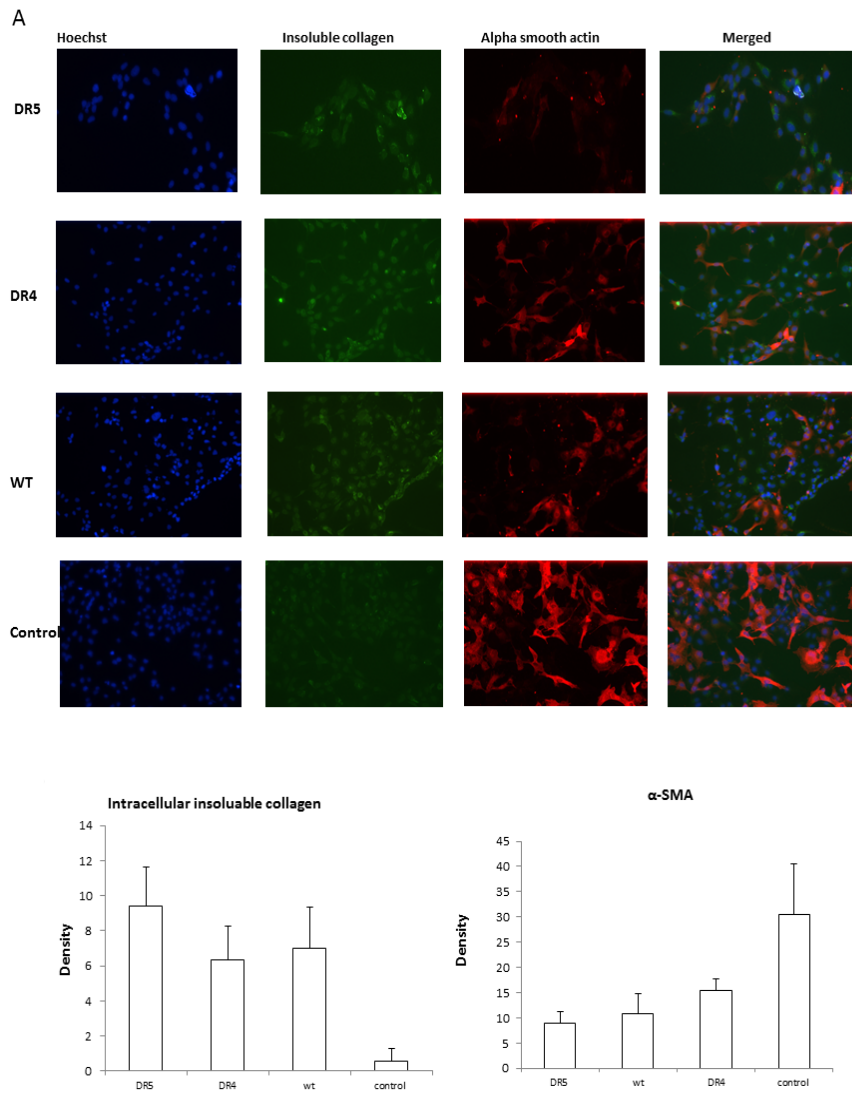
**Figure 1: LX2 cells activation and TRAIL receptor expression profile.** Alpha SMA mRNA and protein expression increase as a sign of activation due to progressive culture of LX2 cells *in vitro* during 9 days(A); (B) mRNA expression profile of TRAIL receptors 1 and 2 in activated LX2 cells by real time PCR; (C) Flow cytometry analysis of TRAIL receptors in LX2 cells. An increase in the expression of surface receptors is associated with the activation of HSCs during culture induced activation. ns; not significant,  $P$  values  $<0.05$  were considered to be significant \*,  $P$  values  $<0.005$  \*\*,  $<0.0005$  highly significant \*\*\*.



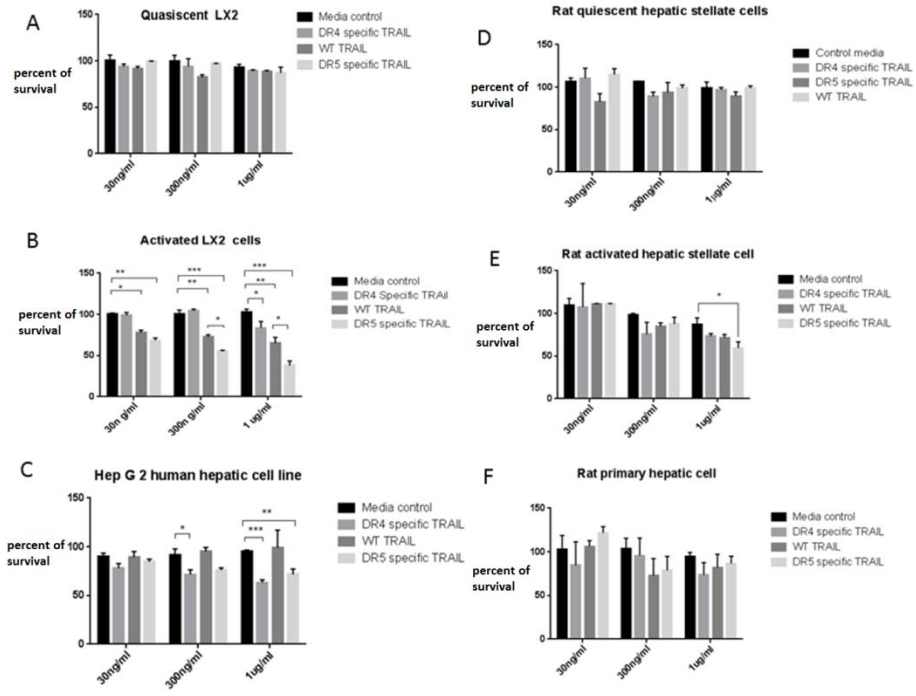
**Figure 2: FACS analysis profiling of TRAIL receptors expression on surface of side population LX2 cells.** LX2 cells were stained with Hoechst 33342 and different TRAIL receptor specific antibodies. Sorted side population cells were separately gated against each specific TRAIL receptors.



**Figure 3: Interfering in LX2 cells collagen production by receptor specific TRAIL variants.** Representative Sircol assay for collagen (A); HSP47 mRNA expression (B) and (C) protein production in LX2 cells treated with three different concentration of TRAIL proteins ( *P* values <0.05 were considered to be significant \*, *P* values <0.005 \*\*, <0.0005 highly significant\*\*\*).

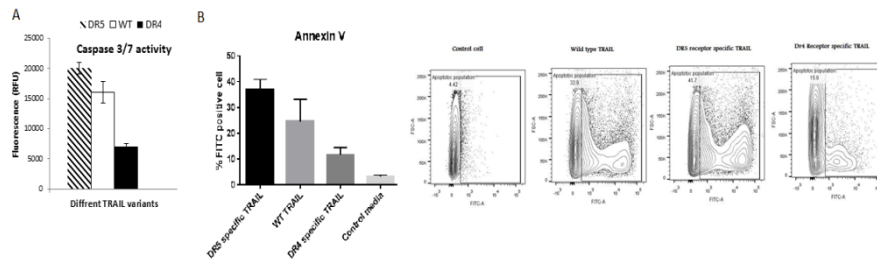


**Figure 4; Immunohistochemistry staining of LX2 cells treated with TRAIL proteins showed a decrease in number of  $\alpha$ SMA positive cells and an increase in intracellular insoluble collagen deposition.** Activated LX2 cells treated with 50 ng/ml of different TRAIL proteins or DMEM (control) for 48 hours and stained for collagen (Green) or  $\alpha$ -SMA (Red) and Hoechst nuclear staining (Blue)(A); integrated density of intracellular insoluble collagen and  $\alpha$ -SMA in LX2 cells affected by different TRAIL or DMEM (control)(B).



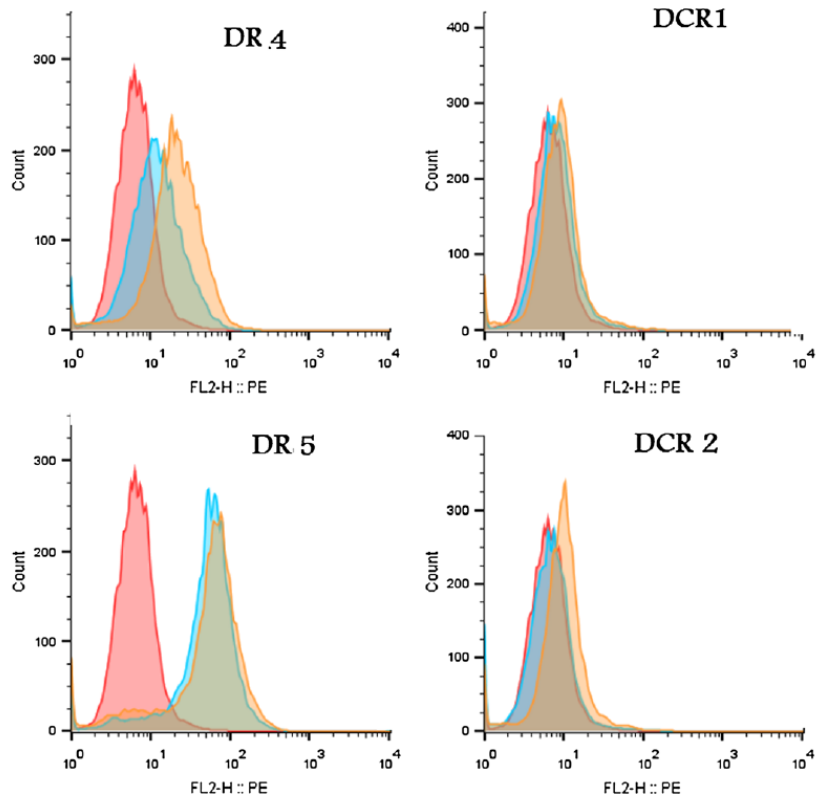
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**Figure 5; TRAIL efficiency in eliminating activated HSCs viability differs depends on TRAIL variants.** MTS viability assay for quiescent LX2 cells (A); activated LX2 cells (B); Hep-G2 cell line (C); quiescent rat primary HSCs (D); activated rat primary HSCs (E); rat hepatocytes (F).



**Figure 6; Comparison of caspase 3/7 enzyme activity and Annexin V in activated HSCs treated with different TRAIL variants.** An increase in the amount of caspase-3/7 activity in LX2 cells (A); treated with TRAIL variants is concurrent with increase in apoptosis marker Annexin V in FACS analysis (B).





**Supplementary Fig. 1** TRAIL receptors expression varies after activation in HSCs. S1 A: TRAIL receptors DR5, DR4 DcR2 and DcR1 expression in **quiescent** (Blue) vs Activated (Brown) HSCs. Isotype control (Pink).