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Orignal Research Paper

Modeling Hepatocellular Toxicity of Enavatuzumab, a Humanized Anti-TweakR Antibody

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Abstract: In a Phase 1 clinical study, treatment with enavatuzumab, a humanized monoclonal antibody to TweakR, resulted in liver toxicity in a subset of patients. The objective of this current study was to evaluate the ability of preclinical studies to predict liver toxicity in humans. Enavatuzumab was evaluated in cynomolgus monkeys, where serum liver enzyme and cytokine levels were measured and histopathology of the liver was performed. TweakR expression was evaluated by immunohistochemistry in healthy human liver and in liver tissues from cancer patients. Enavatuzumab was also evaluated *in vitro* for its impact on human hepatocytes when cultured both alone and with immune cells. Enavatuzumab-treated cynomolgus monkeys exhibited liver enzyme elevations only at the highest dose level (100 mg/kg) and few cytokines were elevated after dosing. Bile duct hyperplasia was observed in the liver but appeared to be partially reversible. Compared with healthy liver, liver tissues from cancer patients exhibited marked elevation of TweakR expression, which was associated with immune cell infiltration. Enavatuzumab treatment of cultured hepatocytes, both in the presence and absence of immune cells resulted in increased cytokine release, but only in the co-cultures were liver enzymes elevated. These results suggest that due to differences in liver architecture between healthy humans and cancer patients, studies in healthy non-human primates may underestimate the potential for liver toxicity in human cancer patients. The use of additional *in vitro* assays in conjunction with *in vivo* studies may better predict the potential impact of a therapeutic agent on the liver in clinical studies.

Keywords: TweakR, Enavatuzumab, Liver Toxicity, Cytokine Release, Cynomolgus

Introduction

Liver toxicity is one of the most common adverse events observed after treatment with pharmaceutical agents (Zimmerman, 2000; Kaplowitz, 2004). The proposed mechanism for this exquisite sensitivity of the liver to small molecule drugs is the accumulation of compounds and their metabolites due to the role of the liver in metabolism and clearance.

A wide range of small molecule chemotherapeutics for oncology treatment, including targeted agents, induce liver toxicity in patients, with varying rates of incidence (Field *et al*., 2008; Field and Michael, 2008). Biologic agents, including antibodies, can also induce liver toxicity, although less prevalently than observed for small molecule therapies. The nature of liver toxicity with biologics can vary widely, from asymptomatic elevations of liver enzymes to fatal liver failure (Ogasawara *et al*., 1993; Vonderheide *et al*., 2007; Advani *et al*., 2009; Tolcher *et al*., 2007; Plummer *et al*., 2007). For many such agents that induce liver toxicity, the mechanisms of toxicity are often not well-understood.

Enavatuzumab is a humanized monoclonal antibody targeting TweakR (Fn14, TWEAK receptor, TNFRSF12A) that has been developed for the treatment of patients with solid tumors.

Enavatuzumab induces potent anti-tumor activity in preclinical models through both directly inhibiting the growth of tumor cells and through Antibody-Dependent Cellular Cytotoxicity (ADCC) (Chao *et al*., 2013; Culp *et al*., 2010; Purcell *et al*., 2014). In a Phase 1 study in cancer patients, enavatuzumab treatment every two weeks induced asymptomatic elevations in ALT and AST at all dose levels tested (0.1-1.5 mg/kg) and the Maximum Tolerated Dose (MTD) was found to be 1 mg/kg (Lam *et al*., 2017). Both the frequency and magnitude of liver enzyme elevations increased with antibody dose level, suggesting a dose-response relationship.

In this study, we describe preclinical studies conducted with enavatuzumab, focusing on predicting the potential for liver toxicity in humans. In cynomolgus monkeys, enavatuzumab induced ALT elevations only at the highest dose level tested (100 mg/kg), indicating a disparity in the sensitivities of human cancer patients and non-human primates to enavatuzumab. In contrast to healthy human and cynomolgus liver tissues, liver samples from cancer patients were found to exhibit both inflammation and elevated TweakR expression. Treatment of normal hepatocytes *in vitro* with enavatuzumab induced release of cytokines, but not liver enzymes. However, in a co-culture of hepatocytes and immune cells, enavatuzumab induced both cytokine and ALT/AST release, both of which could be at least partially suppressed by pre-treatment with dexamethasone. Finally, dexamethasone pretreatment did not inhibit the anti-tumor activity of enavatuzumab in xenograft models, suggesting that dexamethasone pretreatment may be a means to suppress liver toxicity without compromising the anti-tumor activity of the antibody.

Materials and Methods

Cells and Reagents

Human lung fibroblasts were purchased from Lonza (Basel, Switzerland). Normal human hepatocytes were purchased from Lonza as fresh cultures in Hepatocyte Complete Media containing hydrocortisone. After receipt, the cells were maintained in Hepatocyte Complete Media without hydrocortisone for 1-3 days before using the cells in assays.

A freshly isolated lung from a cynomolgus monkey (*Macaca fascicularis*) was obtained from Charles River Laboratories (Wilmington, MA). The tissue was finely minced then treated with collagenase I for 6 h at room temperature. Tissue and dissociated cells were then washed several times with media and plated in the presence of 10 ng/mL bFGF and 20 U/mL heparin. Six days later, fibroblasts that had grown out of the tissue were collected and stored at -80°C for future use.

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood collected from healthy human donors at AbbVie Biotherapeutics under an IRB-approved protocol. All experiments using PBMCs were performed with at least two different donors.

Enavatuzumab is a human IgG1 antibody to TweakR described previously (Culp *et al*., 2010). The isotype control antibody was MSL109 (Drobyski *et al*., 1991). Where indicated, antibodies were cross linked with a goat anti-human Fcγ secondary antibody (Jackson Immunoresearch, West Grove, PA).

Immunohistochemistry

Frozen normal human tissues were collected from autopsy samples by Zoion Diagnostic (Shrewsbury, MA) with appropriate IRB approval. Frozen normal cynomolgus tissues were provided by Covance (Madison, WI). Frozen tissues were embedded in OCT medium and cut into 5 μm sections onto slides with a Cryostat (Leica). Enavatuzumab was pre-incubated with a biotinylated secondary antibody for 30 min at room temperature. Human IgG was added to the complex solution immediately prior to incubation with tissue slides to minimize background staining. VECTASTAIN ABC Elite kit (Vector Labs, Burlingame, CA) was used to detect the signal and Hematoxylin Gill#1 (Sigma. St. Louis, MO) was used for counterstaining.

Formalin-Fixed Paraffin Embedded (FFPE) liver biopsies were obtained from three cancer patients in the enavatuzumab Phase 1 study, Subjects 1102, 1111 and 1116 (Lam *et al*., 2017). Two additional FFPE liver samples from patients with cancer were obtained from Discovery Life Sciences (Los Osos, CA). Four of the five cancer patients had previously received chemotherapy treatment. All FFPE samples were stained for TweakR by immunohistochemistry as previously described (Culp *et al*., 2010).

Antibody Treatment of Human and Cynomolgus Lung Fibroblasts

A six point 3-fold serial dilution of enavatuzumab or a human IgG1 isotype control antibody was made in PBS for concentration starting from 300 μg/mL to 1μg/mL. 100 μL of antibodies was added to an ELISA plate and incubated overnight at 4°C. The next day, the antibodies were removed and normal human or cynomolgus lung fibroblasts were added. After an overnight incubation at 37°C, the cell supernatants were collected.

Cytokine and chemokine levels were quantified in supernatants by Luminex® using the Beadlyte® Human Multi-Cytokine Beadmaster™ Kit (Millipore, Billerica, MA). Supernatant (50 μL) was incubated with Beadlyte® Human 22-plex Multi-Cytokine beads, according to the manufacturer's instructions. All samples and standards were assayed in duplicate. Of the 22 cytokines tested, only IL-6, IL-8, MCP-1, MIP-1α and RANTES were detected and the Luminex®

reagents for these five cytokines cross-reacted efficiently with cynomolgus (Giavedoni, 2005).

Toxicity Studies

One-Month Tolerability Study

Purpose-bred cynomolgus monkeys at SNBL USA were administered enavatuzumab via a 30-min intravenous (IV) infusion once weekly for 5 weeks. Two animals (one of each gender) were dosed at each of 4 dose levels: 0 (vehicle), 10, 30, or 100 mg/kg.

Serum samples were collected pre-dose and at 1, 2, 6 and 24 h after the first dose for cytokine measurements. Serum samples were collected for serum biochemistry pre-dose and on D23 (24 h after the 4th dose) during the dosing period. At the conclusion of the study, animals were euthanized and a histopathology evaluation was performed at necropsy by a board-certified veterinarian pathologist.

IL-8, IL-6, GM-CSF, MCP-1, TNFα and IFNγ were quantified in cynomolgus serum samples by Luminex®, according to the manufacturer's instructions (Upstate, Temecula, CA). For most of the cytokines, 50µL of serum, diluted 1:1 with Assay Buffer, was analyzed. Quantification of RANTES was performed similarly, except that the serum was diluted 1:500 with Assay Buffer. All samples and standards were assayed in duplicate.

The tolerability study was conducted under the guidelines of the SNBL Institutional Animal Care and Use Committee (IACUC). The study was not conducted in compliance with the FDA Good Laboratory Practice (GLP) Regulations for Nonclinical Laboratory Studies.

13-Week Toxicity Study

Twenty one naïve male and 21 naïve female purpose-bred cynomolgus monkeys were treated via a 30 min intravenous (IV) infusion once every other week for 13 weeks, for a total of 7 doses, at 0, 3, 10, 30, or 100 mg/kg. The distribution of animals among the groups is shown in Supplemental Table S1. Dosing and recovery phases began on days designated D1 and R1, respectively. Subsequent days in each phase were numbered incrementally. Animals in the Main Study group were sacrificed on D92, 7 days after the 7th dose. For the animals in the Recovery group, a 24-week recovery period (R1-R170) followed administration of the last dose.

Liver enzymes were measured pre-dose, on D37 (8 days after the 3rd dose), D92 and at five time points during the recovery period: R41, R57, R85, R113, R141 and R170. Of the recovery time points, only results at R41 are shown, as no liver enzyme levels were elevated after that time point. Histopathology was conducted on tissues collected at necropsy in both the Main and Recovery groups.

The 13-week toxicity study was conducted at SNBL USA under the guidelines of their IACUC and in full compliance with the Food and Drug Administration GLP regulations for Nonclinical Laboratory Studies (21 CFR, Part 58).

Flow Cytometry

Human and cynomolgus hepatocytes were purchased from BD Biosciences (San Jose, CA). Human and cynomolgus hepatocytes or lung fibroblasts were incubated with primary antibody (enavatuzumab or isotype control antibody) in 50 µL. After washing away unbound primary antibody, cells were incubated with a fluorescein-conjugated goat anti-human IgG antibody (Caltag). Samples were analyzed on a FACScan™ by flow cytometry.

Xenograft Studies

NCI-H358 lung cancer cells were purchased from American Type Culture Collection and the SN12C cell line was obtained from Developmental Therapeutics Program/Division of Cancer Treatment and Diagnosis Tumor Repository at the National Cancer Institute. All cells were cultured as recommended by the supplier.

Human tumor cells, suspended in RPMI, were inoculated subcutaneously into the right flank of 6-week old ICR-SCID mice at 1×10^7 cells per mouse. When the tumors reached approximately 100 mm^3 (length × width × height/2), the animals were randomized into dosing groups. Antibodies were administered at 1 mg/kg, thrice per week and dexamethasone was administered at 5 mg/kg, with the initial dose given 24 h prior to the first antibody dose and subsequent doses given on alternate days to antibody dosing. All animal work was carried out under the NIH guidelines "Guide for the Care and Use of Laboratory Animals" following protocols approved by the AbbVie Biotherapeutics Institutional Animal Care and Use Committee.

Table S1: Dosing groups in the 13-week toxicity study

Dose level (mg/kg)	Number of Animals (male/female)	Necropsy (male/female)	
		Main (D92)	Recovery (R170)
0	5/5	3/3	2/2
3	3/3	3/3	0/0
10	5/5	3/3	2/2
30	3/3	3/3	0/0
100	5/5	3/3	2/2

Sequencing of Cynomolgus Monkey TweakR

Cynomolgus TweakR was amplified from 5' RACE products of cynomogus tissues using PCR primers designed to the published human TweakR/Fn14 sequence (NM_016639.1). The N-terminal portion of cynomolgus TweakR was isolated from cynomolgus PBMC, liver, thymus and thyroid RACE products using the following primers:

Forward 5' GGCGCAGGACGTGCA CTATG
Reverse 5' AGCGGCCGCTGCTGCAGCGCAGCC CAGGC

The C-terminal portion of cynomolgus TweakR was amplified by PCR from a mixture of cynomolgus PBMC, liver, stomach, cerebral cortex and spleen RACE products using the following primers:

Forward5' ATTGGATCCTGGAGACGATGCCGC AGGAG
Reverse 5' GTCTGGGAGGCAGAGACTGGC

The internal portion of cynomolgus TweakR was amplified by PCR from the cynomolgus kidney RACE product using the following primers:

Forward 5' TTCCTGGAGCGCGGACCTGG
Reverse 5' GCCGGTCTCCTCTATGG

A second internal fragment of cynomolgus TweakR was amplified from the cynomolgus spleen RACE product using the following primers:

Forward 5' TCCGTGGCTGGGGAGCAAGC
Reverse 5' GCCGGTCTCCTCTATGG

All PCR products were sub cloned into the TOPA® TA cloning vector (Invitrogen). Multiple clones containing inserts of the appropriate size were sequenced.

Hepatocyte and PBMC Assays

Normal human hepatocytes were maintained in steroid-free media for 24 h. Enavatuzumab or isotype control antibody (10 μg/mL) +/-anti-human secondary antibody (3.3 μg/mL) was added and incubated for an additional 24 h, after which the supernatants were collected.

PBMCs were cultured in the presence of enavatuzumab or a human IgG1 isotype control antibody (10 μg/mL). 24 h later, the plates were centrifuged and supernatants were collected.

Co-culture assay: Normal human hepatocytes in 48-well plates (approximately 2×10^5 cells/well) were maintained in steroid-free media for 24 h, after which freshly-isolated PBMCs (2×10^6 cells/well) were added. Dexamethasone (10 μM) was added to some wells and 24 h later, enavatuzumab or isotype control antibody (10 μg/mL) was added. The supernatants were collected 24 h later.

Transwell® assay: Normal human hepatocytes were maintained in steroid-free media for 3d, after which 10 μM dexamethasone was added to some wells for 24 h prior to addition of the Transwell® containing PBMCs. PBMCs were maintained at 37°C for 24 h after isolation in the presence or absence of 10 μM dexamethasone before adding to the Transwell®. After 4 and 24 h, the number of migrated PBMCs was enumerated by collecting cells from the bottom chamber, staining for CD45 and quantifying with Fluoresbrite Calibration Beads (Polysciences, Warrington, PA) by flow cytometry.

ALT and AST levels were measured in supernatants from the bottom chamber using Max Discovery™ Alanine Transaminase (ALT) Color Endpoint Assay Kit and Max Discovery™ Aspartate Transaminase (AST) Cytotoxicity Assay Kit purchased from Bioo Scientific Corporation (Austin, TX).

Cytokine and chemokine levels were quantified in supernatants from the bottom chamber by Luminex® using the Milliplex MAP Human Cytokine/Chemokine Kit (Millipore). Supernatant (50 μL) was incubated with Beadlyte® Human 26-plex Multi-Cytokine beads, according to the manufacturer's instructions. All samples and standards were assayed in duplicate.

Results

Enavatuzumab Exhibited Similar Binding and Functional Activity on Human and Cynomolgus Cells

Cynomolgus was chosen as the species in which to perform toxicity studies for enavatuzumab because the amino acid sequences of TweakR in human and cynomologus were found to be identical and enavatuzumab exhibited similar binding to normal tissues derived from the two species by immunohistochemistry and flow cytometry (Supplemental Fig. S1 and Fig. 1A). As enavatuzumab is a TweakR agonist antibody and cytokine release is a common downstream effect of activating the TweakR pathway (Burkly *et al*., 2011; Campbell *et al*., 2004), the ability of enavatuzumab to stimulate cytokine release from TweakR-expressing cultured normal human and cynomolgus cells was assessed. Enavatuzumab treatment of human or cynomolgus lung fibroblasts resulted in increased IL-8 levels from cells derived from both species, but IL-6 release only from human cells (Fig. 1B). Other cytokines and chemokines, including MCP-1, MIP-1α and RANTES, were detected in supernatants of lung fibroblasts from both species but were not elevated by enavatuzumab in either species (data not shown).

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          M  A  R  G  S  L  R  R  L  L  R  L  L  V  L  G  L  W  L  A  L  L  R  S  V  A  G  E  Q  A  P  G  T  A
human   1 ATGGCTCGGGGCTCGCTGCGCCGGTTGCTGCGGCTCCTCGTGCTGGGGCTCTGGCTGGCGTTGCTGCGCTCCGTGGCCGGGGAGCAAGCGCCAGGCACCG 100
cyno    1 ATGGCTCGGGGTTCGCTGCGCCGGTTGCTGCGGCTCCTCGTGCTGGGGCTCTGGCTGGCGTTGCTGCGCTCCGTGGCTGGGGAGCAAGCGCCAGGCACCG 100
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            P  C  S  R  G  S  S  W  S  A  D  L  D  K  C  M  D  C  A  S  C  R  A  R  P  H  S  D  F  C  L  G  C
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cyno  101 CCCCCTGCTCCCGCGGCAGTTCCTGGAGCGCGGACCTGGACAAGTGCATGGACTGCGCGTCTTGCAGGGCGCGACCGCACAGCGACTTCTGCCTGGGCTG 200
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human 201 CGCTGCAGCACCTCCTGCCCCCTTCCGGCTGCTTTGGCCCATCCTTGGGGGCGCTCTGAGCCTGACCTTCGTGCTGGGGCTGCTTTCTGGCTTTTTGGTC 300
cyno  201 CGCTGCGGCACCTCCTGCCCCCTTCCGGCTGCTTTGGCCCATCCTTGGGGGCGCTCTGAGTCTGACCTTCGTGCTGGGGCTGCTTTCTGGCTTTCTGGTC 300
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          W  R  R  C  R  R  R  E  K  F  T  T  P  I  E  E  T  G  G  E  G  C  P  A  V  A  L  I  Q
human 301 TGGAGACGATGCCGCAGGAGAGAGAAGTTCACCACCCCCATAGAGGAGACCGGCGGAGAGGGCTGCCCAGCTGTGGCGCTGATCCAGTGA          390
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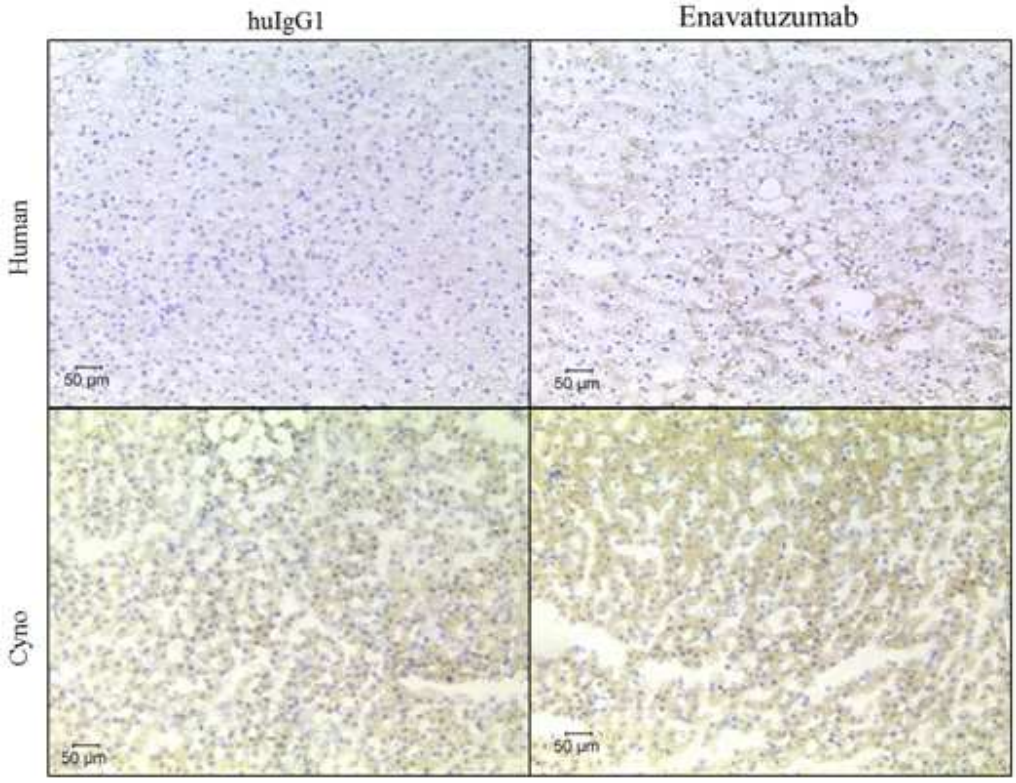

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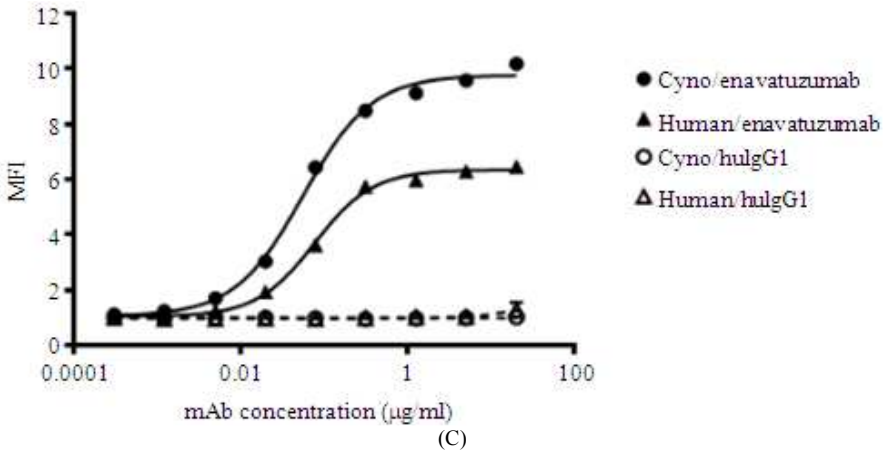

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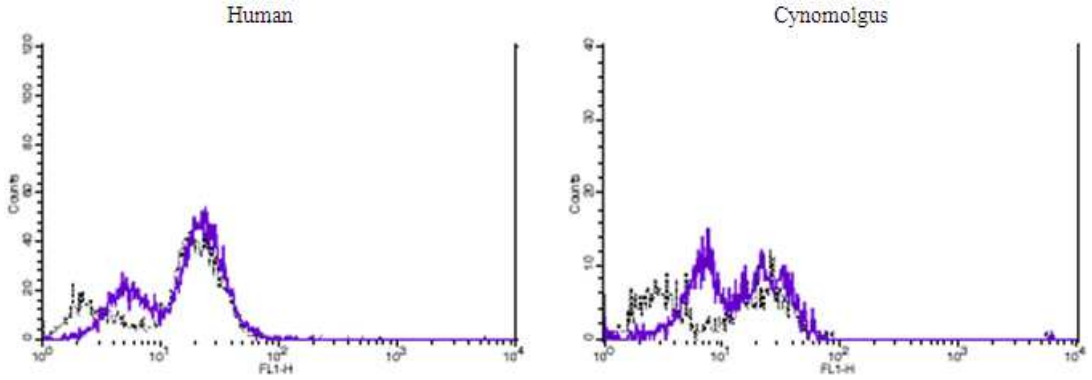
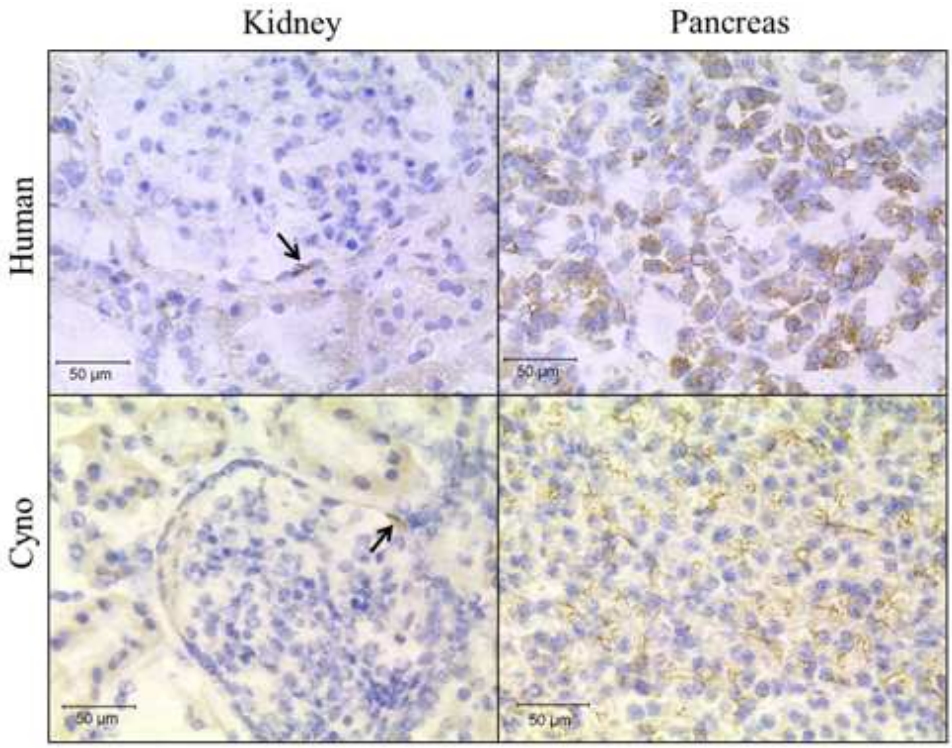


(D)

Fig. S1: Enavatuzumab binding and activity on normal tissues is similar in human and cynomolgus (A) Alignment of the published human (NM_016639.1) and PCR amplified cynomolgus TweakR nucleotide sequences and translated protein sequence (top line). Human and cynomolgus TweakR sequences differ at 6 nucleotides, but upon translation these changes do not lead to any differences at the amino acid level. The start (ATG) and stop (TGA) codons are underlined. * indicates human and cynomolgus TweakR identical nucleotide sequences, (B) Frozen liver tissues from healthy human and cynomolgus donors were stained with enavatuzumab or an isotype control antibody (huIgG1) by immunohistochemistry. Weak nonspecific staining was observed with both the isotype control antibody and enavatuzumab on human and cynomolgus liver tissues. The representative images are shown in 200X magnification (C) Binding of enavatuzumab and an isotype control antibody to normal human and cynomolgus lung fibroblasts was assessed by flow cytometry. Mean Fluorescence Intensity (MFI) is indicated on the Y-axis. Enavatuzumab bound human and cynomolgus cells with similar EC 50 (0.076 and .055 µg /mL, respectively). (D) Enavatuzumab (purple) and an isotype control antibody (black) at 10 µg /mL were incubated with normal human and cynomolgus hepatocytes, after which antibody binding was detected by flow cytometry. Both human and cynomolgus hepatocytes contained two populations of cells, one of which was stained by enavatuzumab in both species



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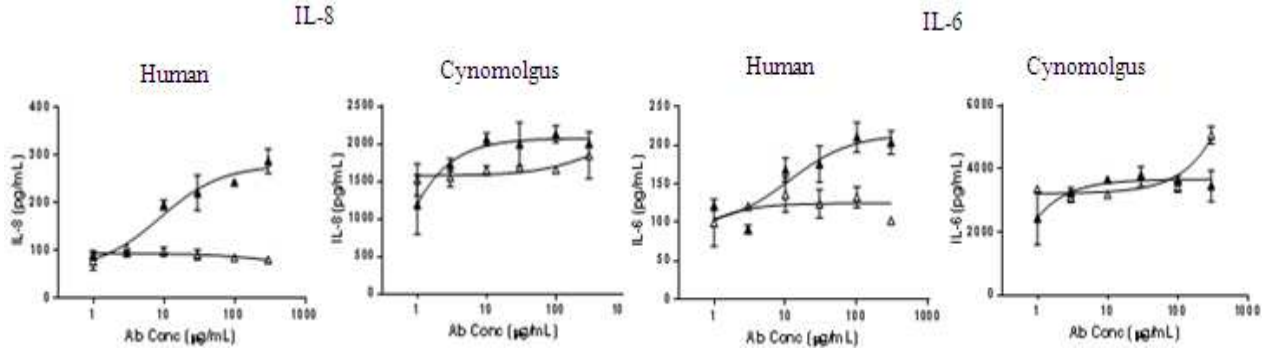

(B)

Fig. 1: Enavatuzumab binding and activity on normal tissues is similar in human and cynomolgus (A) Frozen kidney and pancreas tissues from healthy human and cynomolgus donors were stained with enavatuzumab by immunohistochemistry. Enavatuzumab stained the Bowman's capsule of the glomeruli in the kidney (arrow) in both species and exhibited membranous staining on human and cynomolgus pancreas samples. The representative images are shown in 600x magnification, (B) Cynomolgus and human lung fibroblasts were incubated with immobilized enavatuzumab (closed symbol) or an isotype control antibody (open symbol) for 24 h, after which cytokines and chemokines were measured in the supernatants

Enavatuzumab was Well-Tolerated in Non-Human Primates

To evaluate the impact of enavatuzumab *in vivo*, two toxicity studies were performed in cynomolgus monkeys, a one-month tolerability study and a 13-week GLP toxicity study. In the tolerability study, animals were administered enavatuzumab at one-week intervals, receiving five doses total. As cytokine release has been well-established as a downstream effect of TweakR activation, levels of circulating cytokines were measured in serum from the monkeys after the first dose of enavatuzumab. IL-6 appeared to be elevated in response to enavatuzumab treatment, but only in the mid and high dosing groups, where one animal in each of the 30 mg/kg and 100 mg/kg dosing groups exhibited post-dose IL-6 levels significantly above that in vehicle-dosed animals (Fig. 2A). In contrast, RANTES levels were elevated in post-dose samples in all dosing groups, including the vehicle-dosed group, suggesting that the RANTES elevations were not related to enavatuzumab administration. Other cytokines tested were either not elevated after enavatuzumab treatment or were not detected in any samples (data not shown).

Serum biochemistry was also analyzed in the tolerability study. Compared to pre-dose levels, ALT increased modestly in monkeys dosed with 30 mg/kg, while AST was increased in animals in both the 10 mg/kg and 30 mg/kg dosing groups (Fig. 2B). Only the increases in the 30 mg/kg group were significantly different from the control group and these elevations were within the normal limits. Other serum measurements of liver function, including alkaline phosphatase, bilirubin and albumin levels, showed no indication of liver toxicity induced by enavatuzumab (Supplemental Fig. S2). Histopathology performed on liver samples at the end of the study revealed no changes considered related to enavatuzumab.

In a second toxicity study in cynomolgus monkeys, a 13-week GLP toxicity study, animals were administered enavatuzumab once every two weeks, receiving a total of seven doses. As in the tolerability study, measurements of liver function were largely within the normal range (Fig. 2C). The exception was in the 100 mg/kg dosing group where ALT elevations were observed in two animals on D92 during the dosing period, with elevations of 2.4x and 3.4x over baseline in the two animals. These two animals were in the Main Study group and sacrificed at the end of the dosing period; thus ALT recovery in these animals was not able to be monitored. Also observed in the 100 mg/kg dosing group were minimal decreases in albumin levels in 3 of 5 male animals and 1 of 5 females. Modest AST increases were observed in two animals in the 10 mg/kg dosing group, but no dose-response relationship was observed. ALP and bilirubin levels were not altered in any animals (Supplemental Fig. S2).

In the 13-week toxicity study, the animals in the Main Study group were sacrificed at the end of the dosing period (Day 92), with the remaining animals sacrificed 24 weeks later (R170), having received no additional treatment. From histopathology analysis on the liver tissues, bile duct hyperplasia was noted at necropsy of the Main Study group (Table 1).

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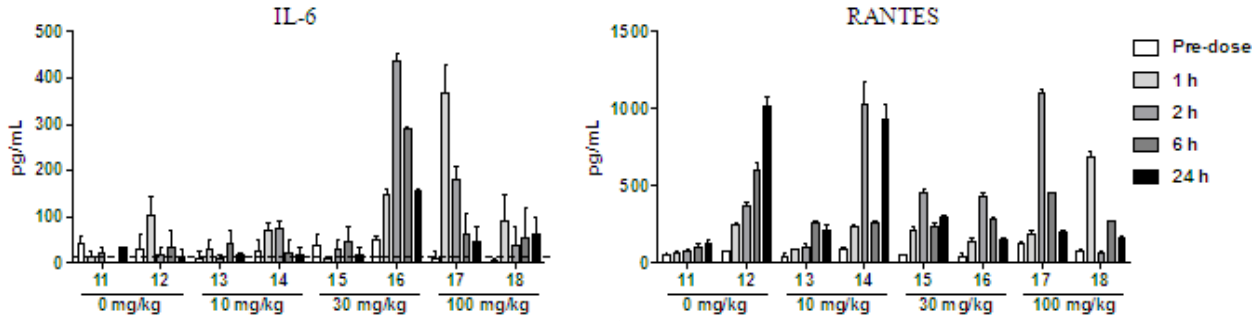

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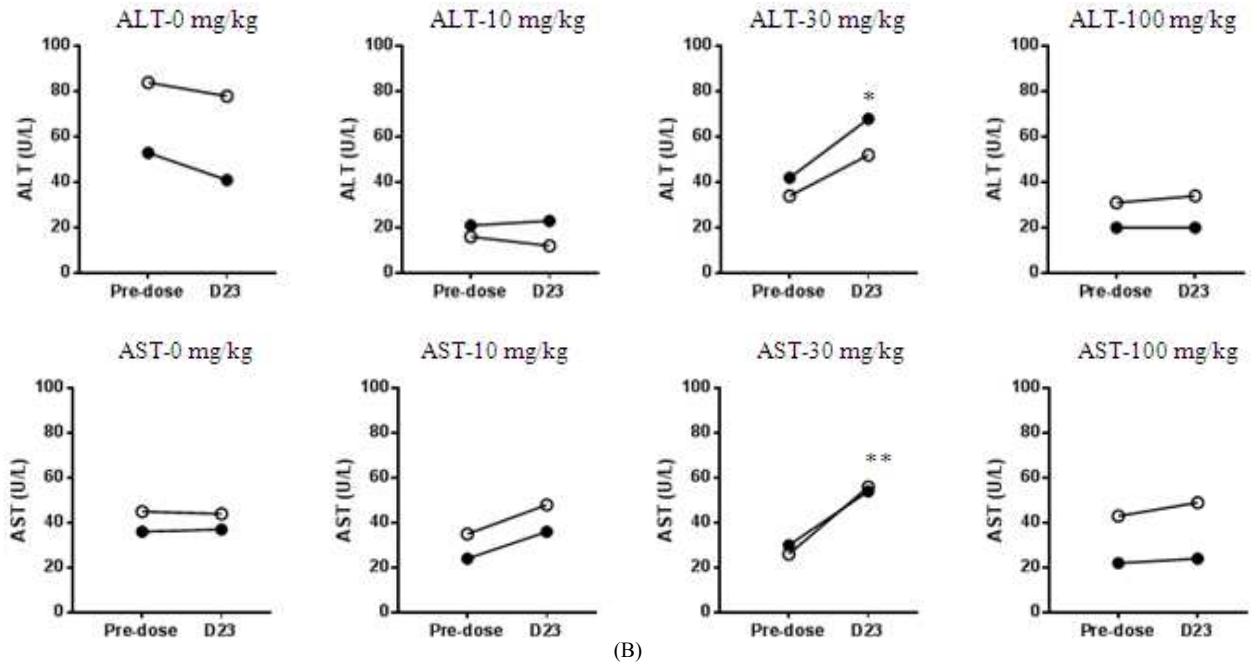

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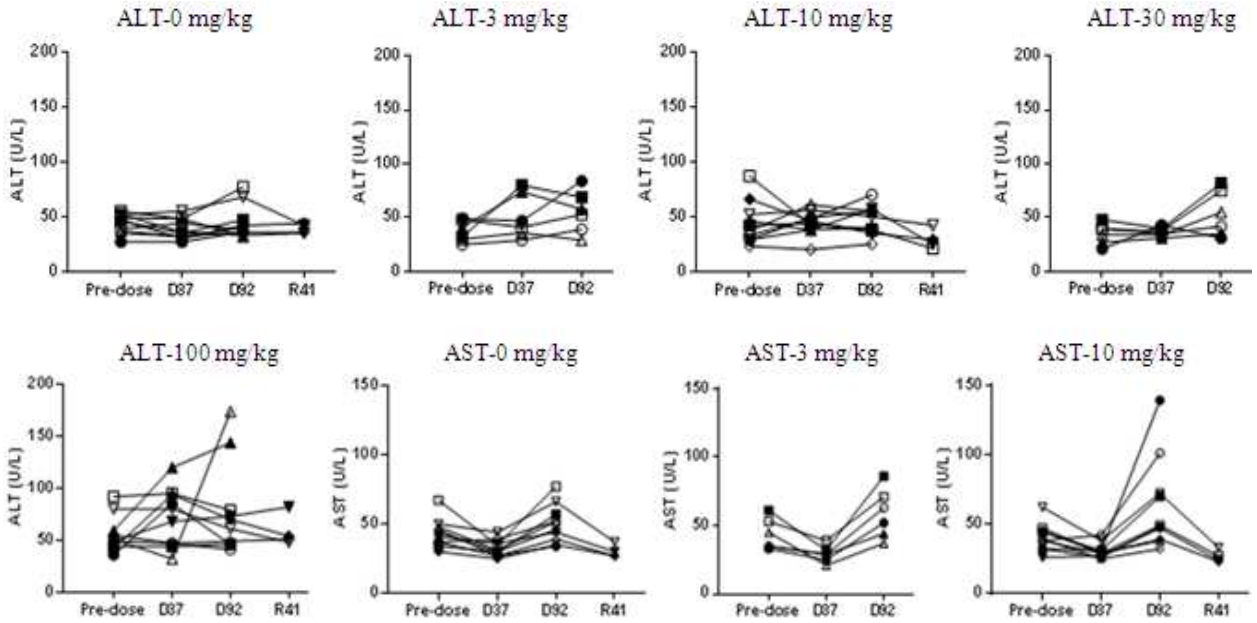
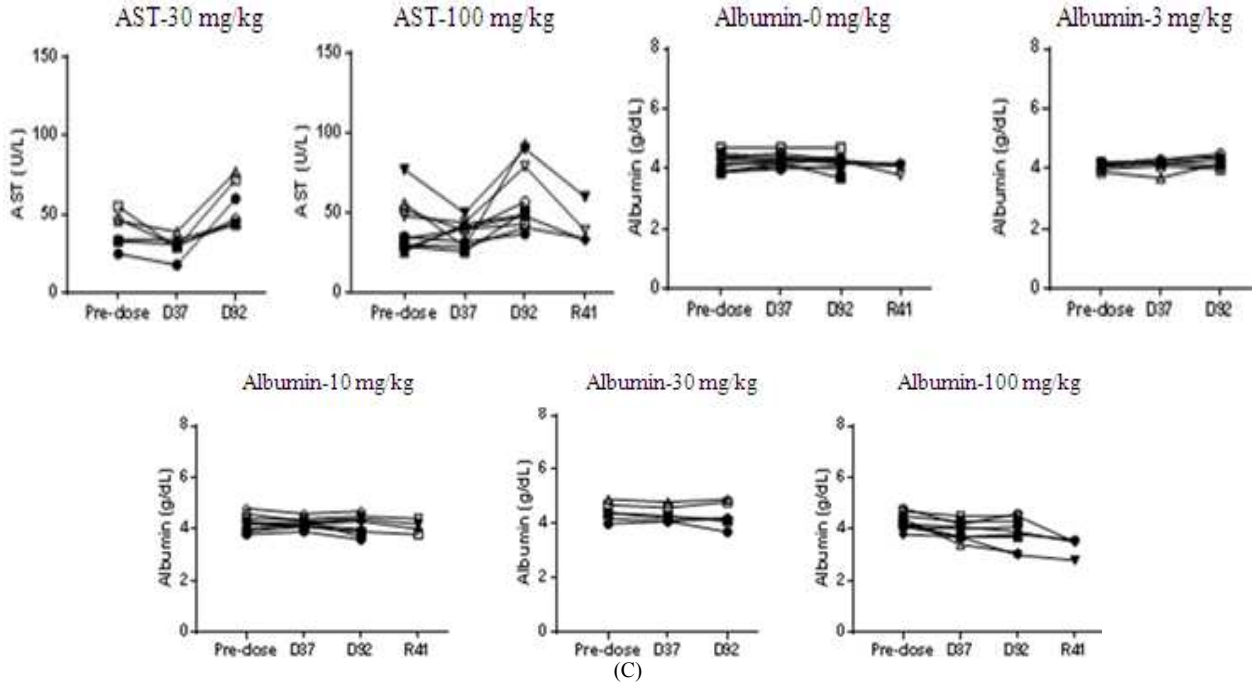
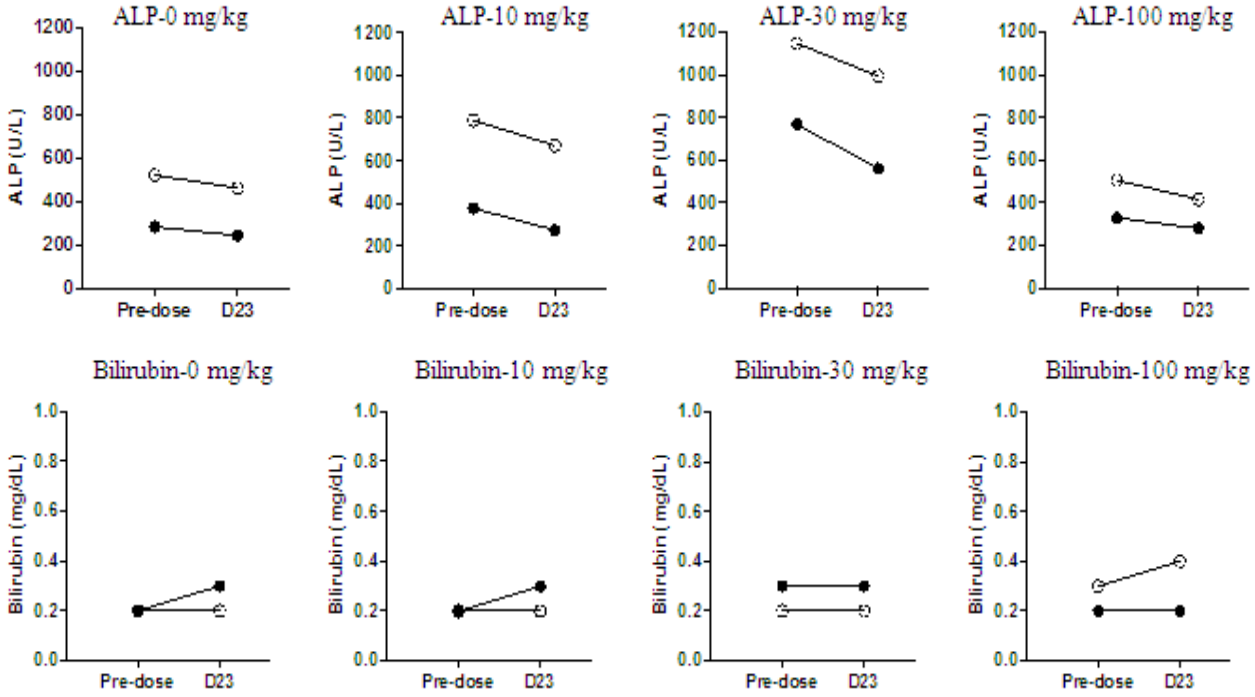




(C)

Fig. 2: Enavatuzumab elicited modest pharmacodynamic changes in cynomolgus monkeys (A) IL-6 and RANTES levels were measured in serum samples collected from cynomolgus monkeys pre - dose and at various time points after the first dose in a one-month tolerability study. The dashed line indicates the lower limit of detection for IL-6. Animal ID numbers are indicated on the X axis; animals 11, 13, 15 and 17 were female and animals 12, 14, 16 and 18 were male, B and C. Alanine Transaminase (ALT) and Aspartate Transaminase (AST) levels were measured in serum samples from cynomolgus monkeys in (B) a one-month tolerability study and (C) a 13 - week toxicity study. Closed symbols: male animals; open symbols: female animals. Increases in ALT and AST in the 30 mg/kg group were statistically significant (*p = 0.01; **p = 0.005)



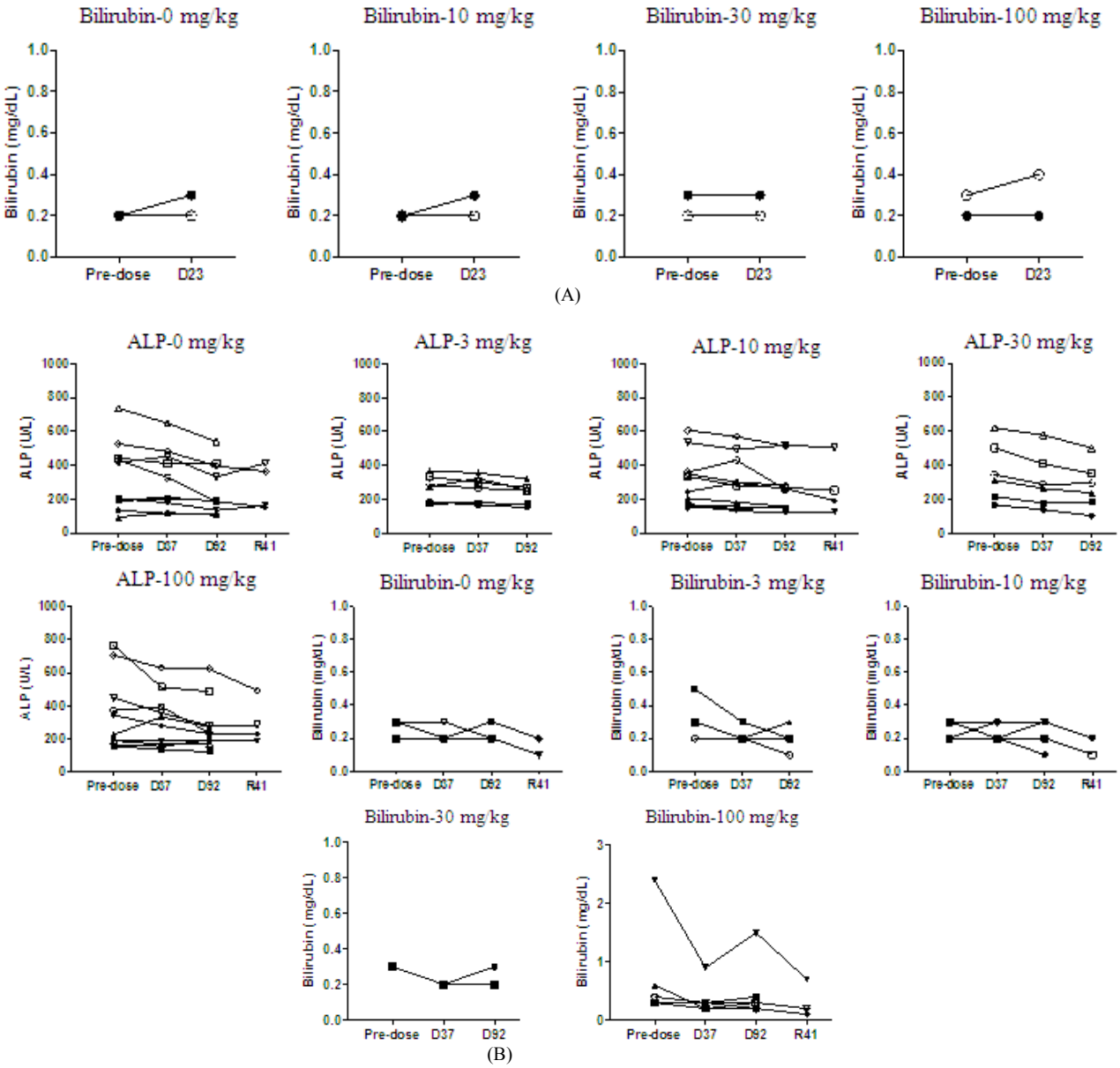


Fig. S2: Additional liver function measurements after enavatuzumab treatment of cynomolgus monkeys, A and B. Alkaline Phosphatase (ALP), bilirubin and albumin levels were measured in serum samples from cynomolgus monkeys in (A) a one-month tolerability study and (B) a 13- week toxicity study. Closed symbols: male animals; open symbols: Female animals. No significant changes in ALP, bilirubin, or albumin levels were observed in enavatuzumab - treated animals in the tolerability study and no significant changes in ALP or bilirubin levels were detected in the 13-week toxicity study

Table 1: Bile duct hyperplasia detected by histopathology in enavatuzumab-treated cynomolgus monkeys

	Necropsy day									
	D92					R170				
Dose level (mg/kg)	N	no findings	minimal	mild	moderate	N	no findings	minimal	mild	moderate
0	6	6	0	0	0	4	4	0	0	0
3	6	3	1	2	0	0				
10	6	3	1	0	2	4	2	2	0	0
30	6	0	4	1	1	0				
100	6	1	2	2	1	4	1	1	1	1

For each dosing group and for each necropsy day (D92, 7 days after the last dose, or R170, 24 weeks after the last dose), the number of animals exhibiting the level of bile duct hyperplasia is indicated. Severity scale: No findings < minimal < mild < moderate < severe

This finding appeared to be related to enavatuzumab treatment, as no animals in the vehicle-dosed group exhibited bile duct hyperplasia. Moreover, the finding appeared to be dose-related, as the incidence and severity increased with antibody dose level. Given that bile duct hyperplasia was observed in all dosing groups, no NOAEL (no observed adverse effect level) was identified in the study.

TweakR Expression was Increased in Liver Samples from Cancer Patients and was Associated with Tissue Inflammation

In a Phase 1 study in cancer patients, elevations in serum cytokines and liver enzymes were observed at significantly lower doses of enavatuzumab than what had been observed in cynomolgus monkeys. Thus we reasoned that liver tissues in human cancer patients may be sensitized to enavatuzumab. To explore this further, we examined liver tissues from five cancer patients and ten healthy human donors. In comparison to liver tissues from healthy donors, liver samples from the cancer patients exhibited marked infiltration of immune cells, accompanied by elevated expression of TweakR, especially notable in the bile ducts (Fig. 3). This finding was observed in all samples from cancer patients, regardless of whether the patients had received prior chemotherapy treatment.

Enavatuzumab Induced Cytokine Release from Hepatocytes and in the Presence of Immune Cells, also Induced Release of Liver Enzymes

To explore further the impact of enavatuzumab on the liver under inflammatory conditions, we established an *in vitro* model to evaluate enavatuzumab-treated hepatocytes in the presence versus the absence of immune cells. Enavatuzumab treatment of cultured hepatocytes had no significant impact on the release of cytokines or chemokines within the timecourse of the assay (Fig. 4A, Supplemental Table S2). However, crosslinking enavatuzumab with secondary antibody increased release of several cytokines and chemokines, including GM-CSF and IL-6. Other cytokines, including IFNγ or TNFα, were not impacted by enavatuzumab. Enavatuzumab treatment did not elevate ALT or AST levels released from the cultured hepatocytes (Fig. 4B).

Table S2: Crosslinking enavatuzumab significantly increased cytokine release from cultured hepatocytes

			+ xlinker	
	huIgG1	Enavatuzumab	huIgG1	enavatuzumab
Eotaxin	28	28	28	32
G-CSF	BLQ	22	49	445
GM-CSF	40	58	77	541*
IFNα2	15	35	28	25
IFNγ	0.4	3	1	2
IL-1α	2	3	2	6
IL-1β	1	3	1	2
IL-2	BLQ	1	BLQ	BLQ
IL-3	BLQ	0.4	BLQ	BLQ
IL-4	BLQ	BLQ	BLQ	BLQ
IL-5	BLQ	BLQ	BLQ	BLQ
IL-6	5	14	24	84*
IL-7	BLQ	1	1	2
IL-8	ALQ	ALQ	ALQ	ALQ
IL-10	BLQ	BLQ	BLQ	BLQ
IL-12p40	3	15	2	2
IL-12p70	1	5	1	2
IL-13	BLQ	0.2	BLQ	BLQ
IL-15	BLQ	1	0.2	1
IL-17	0.5	1	0.4	0.5
IP-10	15	39	38	188*
MCP-1	ALQ	ALQ	ALQ	ALQ
MIP-1α	3	8	19	39
MIP-1β	5	9	25	50
TNF-α	BLQ	0.4	1	5
TNFβ	BLQ	BLQ	BLQ	BLQ

Mean cytokine and chemokine concentrations (in pg/mL) measured in supernatants in a representative experiment *Cytokine levels were significantly increased by enavatuzumab treatment compared to isotype control ($p<0.05$). ALQ: Above the limit of quantification, BLQ: Below the limit of quantification

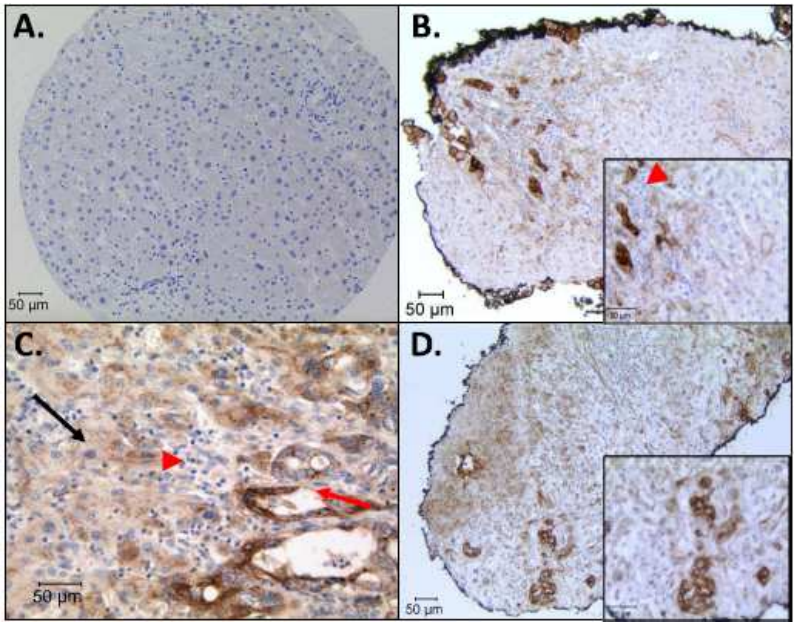
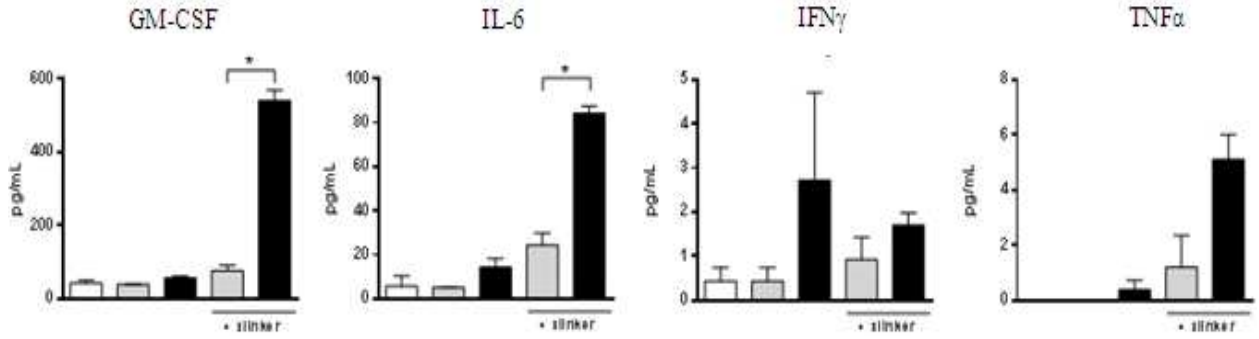
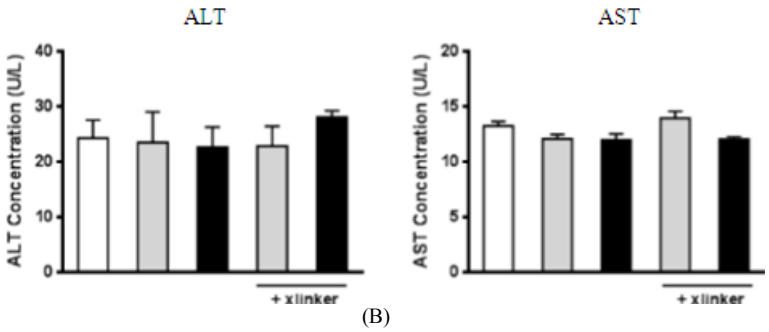


Fig. 3: Liver inflammation in cancer patients is associated with elevated TweakR expression. FFPE liver tissues from a healthy human donor (A) and 3 cancer patients (B-D) were stained for TweakR expression by immunohistochemistry. In B and C, infiltrating immune cells are indicated by red arrowheads. In C, normal hepatocytes and tumor cells are indicated by black and red arrows, respectively. In D, TweakR expression on bile duct is shown in the inset. Panels A, B and D are shown in 200X magnification, panel C is shown in 400X magnification and insets are shown in 600X magnification



(A)



(B)

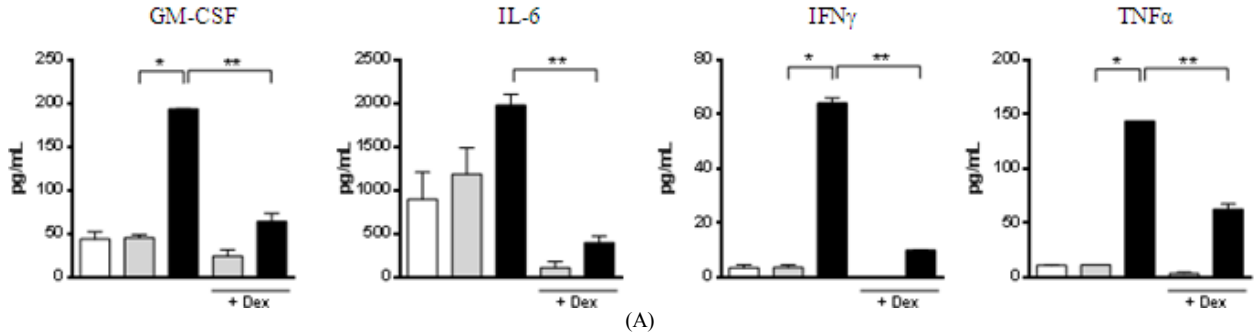
Fig. 4: Enavatuzumab stimulated cytokine and chemokine release from hepatocytes but did not induce release of liver enzymes. Hepatocytes were untreated (white bars) or were incubated with enavatuzumab (black bars) or an isotype control antibody (grey bars) +/- an anti-human Fc crosslinking antibody (+ xlinker) for 24 h, after which supernatants were collected and assessed for (A) cytokines and (B) Alanine Transaminase (ALT) and Aspartate Transaminase (AST) levels. *Enavatuzumab treatment significantly increased cytokine levels ($p<0.05$)

In co-cultures of immune cells and hepatocytes, enavatuzumab treatment significantly elevated the levels of a number of cytokines released from the cultures, including GM-CSF, IFNγ and TNFα (Fig. 5A, Supplemental Table S3). Across multiple experiments using several hepatocyte and immune cell donors, enavatuzumab elicited cytokine release in all co-culture experiments, although the magnitude varied. With a subset of donors, generally in those experiments where higher levels of cytokine release were observed, enavatuzumab treatment of the co-cultures resulted in elevated ALT and AST levels in the supernatants (Fig. 5B).

Table S3: Enavatuzumab induced cytokine release in co-cultures of hepatocytes and immune cells

			+ Dexamethasone	
	huIgG1	enavatuzumab	huIgG1	enavatuzumab
Eotaxin	41	67	28	51*†
G-CSF	1841	2557	1040	1704
GM-CSF	45	194*	24	64†
IFNα2	11	11	3	12
IFNγ	4	64*	0.1	10*†
IL-1α	129	348*	105	262*†
IL-1β	45	91	4	9
IL-2	2	2	0.1	0.2
IL-3	BLQ	BLQ	BLQ	BLQ
IL-4	BLQ	BLQ	BLQ	BLQ
IL-5	BLQ	BLQ	BLQ	BLQ
IL-6	1185	1980	105	396†
IL-7	6	11	BLQ	4
IL-8	ALQ	ALQ	ALQ	ALQ
IL-10	BLQ	3*	12	12
IL-12p40	6	29*	1	10
IL-12p70	2	30*	0.3	12*†
IL-13	BLQ	7	BLQ	BLQ
IL-15	BLQ	BLQ	BLQ	BLQ
IL-17	1	1	0.2	0.5
IP-10	5053	ALQ	4828	ALQ
MCP-1	5639	ALQ	732	ALQ
MIP-1α	9	197*	BLQ	34†
MIP-1β	75	3242*	47	1909*†
TNF-α	11	144*	3	63*†
TNFβ	BLQ	BLQ	BLQ	BLQ

Mean cytokine and chemokine concentrations (in pg/mL) measured in supernatants in a representative experiment *Cytokine levels were significantly increased by enavatuzumab treatment compared to isotype control ($p<0.05$) †Cytokine levels in enavatuzumab-treated samples were significantly reduced by dexamethasone pre-treatment ($p<0.05$), BLQ: Below the limit of quantification



(A)

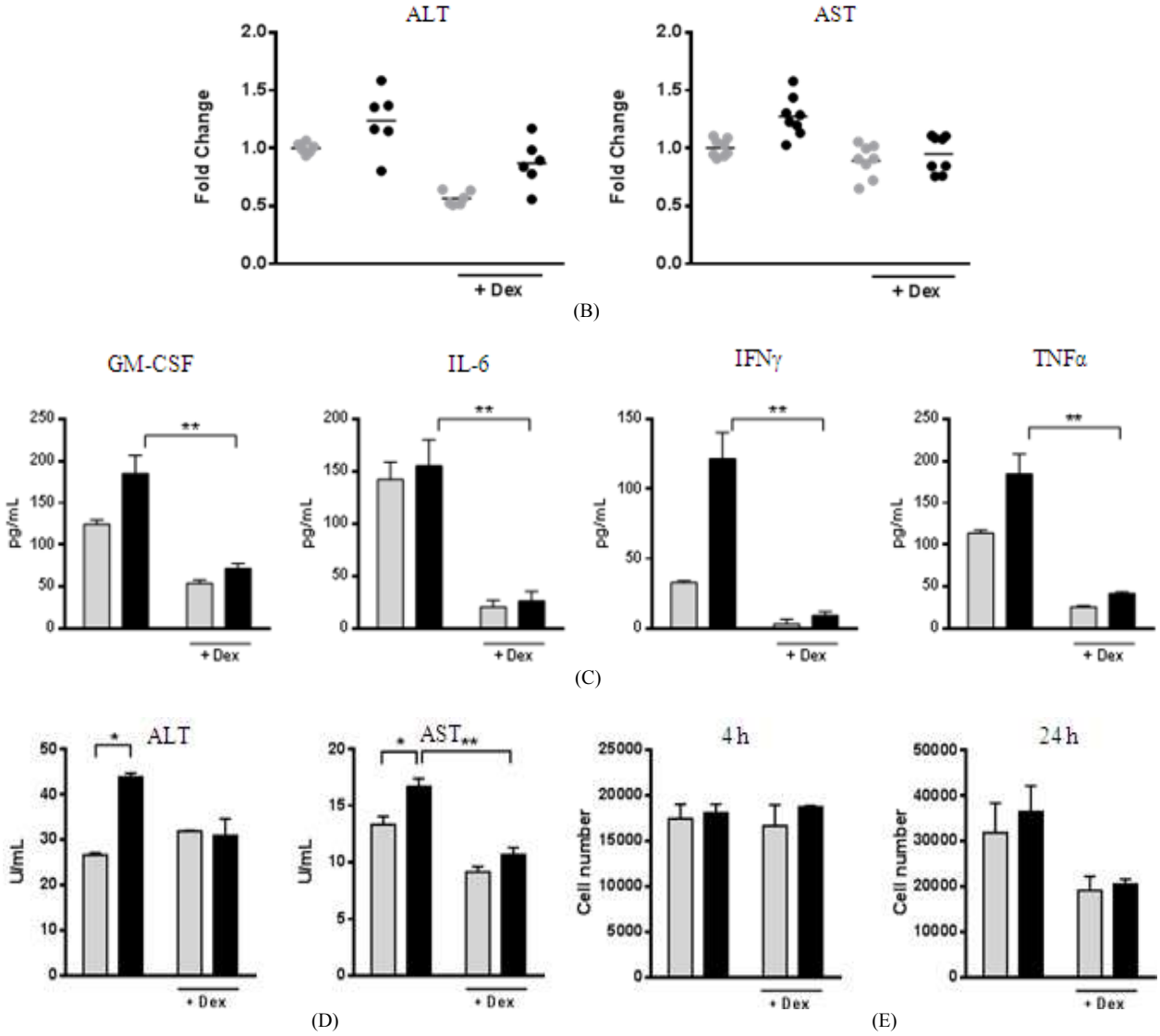


Fig. 5: Enavatuzumab stimulated release of cytokines and liver enzymes in hepatocytes cultured with immune cells (A) Hepatocytes and PBMCs were co-cultured for 24 h in the presence of enavatuzumab (black bars), an isotype control antibody (grey bars), or no antibody (white bars), after which cytokine levels were quantified in the supernatants. Samples treated with dexamethasone prior to antibody treatment are indicated, (B) Supernatants from hepatocyte: PBMC co-cultures were analyzed for ALT and AST levels 24 h after treatment with enavatuzumab (black circles) or an isotype control antibody (grey circles). Each circle represents a different hepatocyte and/or PBMC donor. The data were normalized to the mean of the isotype treated group (without dexamethasone) and fold change is indicated (C-E) Hepatocytes in the lower chamber and PBMCs in the upper chamber of a Transwell® assay were incubated with enavatuzumab (black bars) or isotype control antibody (grey bars) for 4h or 24 h, after which (C) the levels of cytokines were measured in the supernatants; (D) ALT and AST levels were measured; or (E) the number of immune cells that had migrated to the lower chamber was quantified. For dexamethasone - treated samples, hepatocytes and PBMCs were treated separately with 10 μM dexamethasone prior to placement in the Transwell® and addition of antibody. * Enavatuzumab treatment significantly increased cytokine or liver enzyme levels ($p<0.05$). ** Dexamethasone treatment significantly decreased cytokine or liver enzyme levels ($p<0.05$)

Enavatuzumab treatment of cultured immune cells alone did not elicit cytokine release (Supplemental Table S4), suggesting that the impact of enavatuzumab on the hepatocyte: PBMC co-cultures was a result of enavatuzumab binding and activating TweakR expressed on the hepatocytes. However, cytokines and/or chemokines released by the hepatocytes in response to enavatuzumab treatment likely activated the immune cells to elicit

secondary effects, resulting in the elevated pro-inflammatory cytokines and ALT/AST release. To explore further the potential relationship between cytokines and/or chemokines and liver enzyme release, hepatocyte: PBMC co-cultures were pre-treated with dexamethasone to suppress cytokine release. Enavatuzumab treatment of dexamethasone-treated samples resulted in significantly reduced levels of cytokinesand AST levels (Fig. 5A-B, Supplemental Table S3).

Enavatuzumab was also evaluated on hepatocyte and PBMC cultures in a Transwell® assay, where the immune cells in the upper chamber were able to migrate towards enavatuzumab-treated hepatocytes in the lower chamber. In this assay, enavatuzumab treatment resulted in a trend toward elevated cytokine levels but did not reach significance (Fig. 5C, Supplemental Table S5). As observed in the co-culture assay, enavatuzumab stimulated ALT and AST release in a subset of donors in the Transwell® assay (Fig. 5D) and dexamethasone pre-treatment of the cells significantly suppressed cytokine and ALT/AST elevations. Quantification of the migrating immune cells revealed that enavatuzumab treatment had no impact on the migration of immune cells toward hepatocytes (Fig. 5E). However, dexamethasone pretreatment resulted in a significant reduction in the number of the baseline immune cells that had migrated after 24 h.

Dexamethasone did not Impact the Anti-Tumor Activity of Enavatuzumab

The finding that dexamethasone effectively reduced enavatuzumab-stimulated cytokine/chemokine and ALT/AST levels *in vitro* suggested that steroid pre-treatment may be a means to alleviate enavatuzumab-induced liver toxicity in vivo. Thus, we evaluated the impact of dexamethasone on the anti-tumor activity of enavatuzumab in mouse xenograft models. As previously described, enavatuzumab elicits potent anti-tumor activity through two mechanisms of action: direct inhibition of tumor cell growth and antibody-dependent cellular cytotoxicity (ADCC) (Culp *et al*., 2010). We evaluated the impact of dexamethasone on the anti-tumor activity of enavatuzumab in two xenograft models: H358, an ADCC-independent model and SN12C, an ADCC-dependent model (Culp *et al*., 2010; Purcell *et al*., 2014). In both models, dexamethasone modestly inhibited tumor growth and either had no impact on enavatuzumab activity or modestly enhanced the anti-tumor activity of enavatuzumab (Supplemental Fig. S3).

Table S4: Enavatuzumab did not induce cytokine release from PBMCs

	huIgG1	enavatuzumab
Eotaxin	26	26.0
GM-CSF	8	8.0
IFNγ	13	10.0
IL-1α	31	35.0
IL-1β	17	10.0
IL-2	6	4.0
IL-3	5	5.0
IL-4	6	5.0
IL-5	4	3.5
IL-6	92	123.0
IL-7	4	4.0
IL-8	2398	3306.0
IL-10	35	32.0
IL-12p40	178	171.0
IL-12p70	6	5.0
IL-13	2	2.0
IL-15	7	6.0
IP-10	58	130.0
MCP-1	52	69.0
MIP-1α	137	197.0
RANTES	12250	12880.0
TNFα	6	5.0

Mean cytokine and chemokine concentrations (in pg/mL) measured in supernatants in a representative experiment

Table S5: Enavatuzumab induced cytokine release from Transwell® assays containing hepatocytes and PBMCs

			+ Dexamethasone	
	huIgG1	enavatuzumab	huIgG1	enavatuzumab
Eotaxin	163	187	95	129
G-CSF	84	141	46	46
GM-CSF	125	185	54	72†
IFNα2	228	219	115	155
IFNγ	33	122	BLQ	9†
IL-1α	219	247	148	223
IL-1β	36	54	14	14
IL-2	19	37	7	6
IL-3	BLQ	BLQ	BLQ	BLQ
IL-4	34	57	14	22
IL-5	11	24	4	BLQ
IL-6	142	155	20	26†
IL-7	38	48	19	23
IL-8	43501	44656	8435	9893
IL-10	BLQ	17	BLQ	BLQ
IL-12p40	11	26	BLQ	6
IL-12p70	17	38	6	8
IL-13	38	59	15	15
IL-15	20	30	13	15
IL-17	4	4	BLQ	1
IP-10	390346	1150337	14670	248075†
MCP-1	18946	26245	625	1174†
MIP-1α	318	533	BLQ	205*†
MIP-1β	969	2451	370	845
TNFα	114	185	26	42†
TNFβ	24	39	BLQ	BLQ

Mean cytokine and chemokine concentrations (in pg/mL) measured in supernatants in a representative experiment *Cytokine levels were significantly increased by enavatuzumab treatment compared to isotype control ($p<0.05$) †Cytokine levels in enavatuzumab-treated samples were significantly reduced by dexamethasone pre-treatment ($p<0.05$), BLQ: Below the limit of quantification

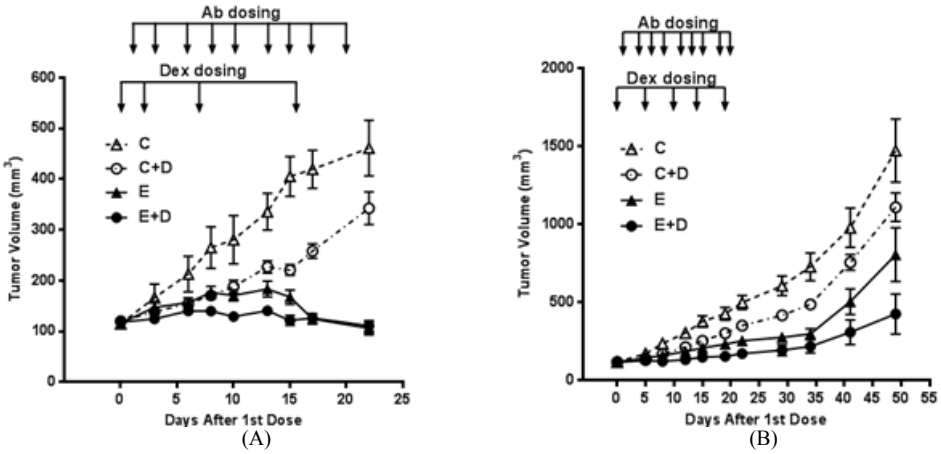


Fig. S3: Dexamethasone did not impact the anti-tumor activity of enavatuzumab, ICR-SCID mice bearing (A) NCI-H358 or (B) SN12C xenograft tumors were treated with Enavatuzumab (E) or an isotype control antibody (C) (1 mg/kg) +/- dexamethasone (D) (5 mg/kg). Dosing days are indicated on each graph and tumor volumes on each day of measurement are indicated as the mean +/- SEM

Discussion

This report describes the preclinical studies evaluating the mechanism of liver toxicity induced by enavatuzumab. No liver toxicity was observed in the one-month tolerability study in cynomolgus monkeys and in the 13-week toxicity study, enavatuzumab induced ALT elevations only at the highest dose level

tested, 100 mg/kg. However, in this latter study, bile duct hyperplasia was observed at all dose levels. This histopathology finding is consistent with a previous report describing the impact of activating the TWEAK/TweakR pathway on the liver in mice. TWEAK transgenic mice exhibit hyperplasia of oval cells, which are liver progenitor cells, but the animals do not exhibit liver enzyme elevations (Jakubowski *et al*., 2005). In the same study, the authors demonstrated that ectopic expression of TWEAK in the liver of adult mice resulted in oval cell and biliary duct hyperplasia without significant liver damage. While activating the TweakR pathway in cynomolgus and mouse exhibited similar signs of liver toxicity by histopathology, these preclinical studies greatly underestimated the sensitivity of human cancer patients to liver toxicity induced by enavatuzumab, as measured by ALT and AST elevations. Whether enavatuzumab induced hyperplasia in the livers of the patients in the Phase 1 study is unknown, as post-dose liver biopsies were not available.

One possible explanation for the disparity in sensitivities of human and cynomolgus to enavatuzumab is innate differences in the sensitivities of the two species to the antibody. Using several cynomolgus tissue sources we found the amino acid sequences between human and cynomolgus to be identical. This is in contrast to a previous report describing cynomolgus TweakR as 98% identical to human TweakR (Zheng and Burkly, 2008). However, enavatuzumab bound TweakR in both species similarly and induced cytokine release in the two species. Another and perhaps more likely, explanation for the increased sensitivity of humans to enavatuzumab is that the architecture of the liver in cancer patients is altered, as observed by infiltration of immune cells and elevated TweakR expression, either of which may contribute to enavatuzumab sensitivity.

In the liver tissues of cancer patients, the areas of inflammation in the liver were accompanied by elevated TweakR expression. We observed a similar increase in TweakR expression in a liver sample from a patient with liver inflammation that exhibited bile duct dilatation and mild fibrosis but not from a patient who exhibited non-inflammatory angiomyolipoma (data not shown). The elevation of TweakR expression in inflammatory tissues has also been described in multiple disease states, including lupus, rheumatoid arthritis, psoriatic arthritis and multiple sclerosis (Lu *et al*., 2011; van Kuijk *et al*., 2010; Dharmapatni *et al*., 2011; Serafini *et al*., 2008).

In experiments designed to explore the mechanism of enavatuzumab-induced liver toxicity, co-cultures of hepatocytes and immune cells were used to model the impact of enavatuzumab on cytokine and ALT/AST release. A range of methods for evaluating liver toxicity have previously been described, including use of liver tissue slices and 2D or 3D co-cultures of hepatocytes with other cell types (Soldatow *et al*., 2013; Lake and Price, 2013; Sahi *et al*., 2010; Gomez-Lechon *et al*., 2010). In such models, where hepatocytes are co-cultured with other cell types, the relevant cell-cell interactions that occur *in vivo* are replicated *in vitro*. In the enavatuzumab Phase 1 study, liver toxicity was largely limited to ALT/AST elevations, suggesting that the toxicity elicited by enavatuzumab was likely due to direct stimulation of hepatocytes, consistent with the expression of TweakR on hepatocytes in cancer patients. Given the likely direct effect of enavatuzumab on hepatocytes and the inflammation observed in liver samples from cancer patients, a co-culture of hepatocytes and PBMCs was able to replicate some of the relevant cell-cell interactions in the livers of cancer patients. However, a more complex model, such as the use of tissue slices from cancer patients, would contain additional cell types and the effect of enavatuzumab in such a system would be interesting to explore.

Interestingly, cultured normal hepatocytes expressed TweakR, unlike hepatocytes that reside in non-diseased livers. The elevated expression of TweakR on cultured hepatocytes may be a result of the culture conditions, as growth factors in culture media have been shown to upregulate TweakR expression (Wiley *et al*., 2001). Fortuitously, TweakR expression on hepatocytes cultured in the absence of immune cells provided the opportunity to evaluate the direct effect of enavatuzumab treatment of hepatocytes.

Immune cells provide multiple potential mechanisms for impacting the functional activity of enavatuzumab on hepatocytes, one of which is to crosslink the antibody through Fc receptors expressed on the immune cells. Mimicking that function with a secondary antibody dramatically increased the level of cytokines released by enavatuzumab; this finding is consistent with increased functional activity of enavatuzumab and other TNFRSF agonist antibodies when crosslinked with a secondary antibody or Protein A (Chao *et al*., 2013; Salzmann *et al*., 2013; Chuntharapai *et al*., 2001; White *et al*., 2013; Li *et al*., 2006; Dhein *et al*., 1992; Chodorge *et al*., 2012). However, crosslinking the antibody did not increase ALT/AST release, which was only observed when immune cells were present. In addition, in the presence of immune cells, the type of cytokines induced by enavatuzumab was altered. Crosslinked enavatuzumab resulted largely in the release of the chemokines GM-CSF, IL-6 and IP-10; however in co-cultures of hepatocytes and immune cells, additional chemokines and the pro-inflammatory cytokines, IFNγ and TNFα, were strikingly enhanced by enavatuzumab treatment. Such a qualitative change may be due, at least in part, to an allogeneic response, as the hepatocytes and PBMCs were not from matched donors. The mechanism of liver toxicity induced by

enavatuzumab in co-cultures of hepatocytes and immune cells may be a result of ADCC or may be indirect, possibly through a cytokine-mediated effect. Our finding that dexamethasone inhibited enavatuzumab-dependent ALT/AST increases suggests that the hepatocellular toxicity was mediated at least in part by cytokines. These results indicate that immune cells likely mediate multiple functions to induce liver toxicity with enavatuzumab.

In the *in vitro* assays, while experiments across all PBMC and hepatocyte donors exhibited some elevations in secreted cytokines upon enavatuzumab treatment, stimulation of ALT and AST release was observed only in a subset of donors and generally in experiments where higher levels of cytokines were released. This varied response to enavatuzumab across human donors reflected the variation of liver toxicity observed in cancer patients in the Phase 1 study. Of the three patients from the Phase 1 study whose liver biopsies were tested for TweakR expression by IHC, all showed increased TweakR expression and inflammation, but only one patient had experienced liver enzyme elevations after enavatuzumab treatment (Lam *et al*., 2017). Thus, increased TweakR expression and/or inflammation may contribute to sensitivity to enavatuzumab, but additional factors are also likely involved in inducing liver toxicity. In the Phase 1 study, a number of cytokines were elevated in the serum of a subset of patients after enavatuzumab treatment; however, cytokine and ALT/AST elevations were not correlated (Lam *et al*., 2017). As multiple normal tissues express TweakR, the post-dose cytokine elevations may have arisen from the liver or other TweakR-positive tissues.

Liver toxicity is less commonly observed with biologics agents than with small molecule chemotherapy agents (Baldo, 2013; Saez *et al*., 2012). A notable exception is the TNF Receptor Super Family (TNFRSF), where agonist antibodies to multiple TNFRSF members have been found to induce liver toxicity with a range of incidence and severity. Agonist antibodies to CD95 (Fas) rapidly produce profound and fatal liver failure in mice (Ogasawara *et al*., 1993) and agonist antibodies to CD137 (4-1BB), CD40 and the TRAIL receptors DR4 and DR5, have all exhibited liver toxicity in humans, as defined by ALT and/or AST elevations in a subset of patients treated with the antibodies (Vonderheide *et al*., 2007; Advani *et al*., 2009; Tolcher *et al*., 2007; Plummer *et al*., 2007; Ascierto, 2010; Camidge *et al*., 2010). In the case of the CD137 antibody, which also produced Grade 4 hepatitis in some patients, the adverse events were not predicted in preclinical studies in cynomolgus monkey, but CD137 antibodies did induce liver toxicity in mice (Ascierto *et al*., 2010; Niu *et al*., 2007). The mechanisms by which the different agonists induce liver toxicity likely depend on the cell types on which the receptors and/or ligands are expressed. For several of the TNFRSF members, receptor expression has been reported on hepatocytes within either healthy liver and/or in liver disease states (Afford *et al*., 1999; Leifeld *et al*., 1999; Volkmann *et al*., 2007; Galle *et al*., 1995), while for other TNFRSF members, other cells within the liver have been shown to express the receptors (Niu *et al*., 2007; Leifeld *et al*., 1999; Schwabe *et al*., 2001).

Of note, only agonist antibodies targeting TNFRSF members induce liver toxicity; antagonist or blocking antibodies have not exhibited such an effect (Croft *et al*., 2013). Thus, the activation of downstream signaling pathways and not simply receptor binding, is key to inducing liver toxicity. TweakR signals through a functional interaction between its cytoplasmic domain and one or more intracellular TRAF proteins, a signaling mechanism common to other TNFRSF members, including CD137 and CD40. One signaling pathway downstream of the TRAFs and activated by the majority of TNFRSF members is NFκB; up regulation of this pathway often results in increased production of pro inflammatory cytokines (Younes and Kadin, 2003; Napetschnig and Wu, 2013). That liver toxicity has been observed with multiple TNFRSF members in cancer patients, together with our finding that the liver tissues of cancer patients often harbor inflammation, suggests that cytokines may be involved in inducing liver toxicity across this family of molecules, as has been implicated for CD137 and CD40 (Niu *et al*., 2007; Kimura *et al*., 2006). In a disease state, such as cancer, where the liver has experienced significant insult and inflammation, changes in the expression of the receptors and/or ligands likely increases the liver's sensitivity to cytokines. Given that a common downstream effect of TNFRSF stimulation is cytokine release, therapeutic agonists to this family of receptors have the potential for inducing liver toxicity in some settings; employing preclinical methods to evaluate the impact of the agent on the liver can be useful for determining the potential for inducing liver toxicity.

Conclusion

The presence of immune cells in liver tissue can provide an additional mechanism by which a drug induces liver toxicity in cancer patients, which may not be predicted from preclinical toxicity studies in healthy animals. Including studies to evaluate the impact of an agent on hepatocytes in the presence of immune cells may provide a more comprehensive analysis of the potential for liver toxicity in cancer patients.

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Author's Contributions

Donghee Choi: Design and execution of *in vitro* experiments, analysis and interpretation of results, drafting of manuscript, revision and approval of final manuscript.

Yanhong Zhu: Design of cynomolgus monkey toxicity studies and interpretation of results**.**

Debra T. Chao: Design of IHC analysis, interpretation of data, drafting manuscript, revision and approval of final manuscript

Mien Sho: Analysis of TwerkR expression by IHC and interpretation of results

Susan Rhodes: Analysis and determination of cynomolgus monkey TweakR DNA and protein sequence

Melvin Fox: Execution of mouse xenograft studies.

Gary C. Starling: Design of experiments, revision and approval of final manuscript

Patricia A. Culp: Conception and design of experiments, analysis and interpretation of data, drafting initial version of the manuscript, revision and approval of the final version of the manuscript.

Conflict of Interest

D Choi, DT Chao, M Sho, S Rhodes and M Fox are employees of AbbVie. Y Zhu, GC Starling and PA Culp were employees of AbbVie at the time of the study. The design, study conduct and financial support for this research were provided by AbbVie.

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