Antibody-Targeted Myofibroblast Apoptosis Reduces Fibrosis During Sustained Liver Injury

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Background /Aims: Myofibroblast apoptosis promotes the resolution of liver fibrosis. However, retaining macrophages may enhance reversal. The effects of specifically stimulating myofibroblast apoptosis in vivo was assessed. Methods: An antibody (C1-3) to an extracellular domain of a myofibroblast membrane protein was injected as a fluorescent- or gliotoxin-conjugate into mice with liver fibrosis. Results: C1-3 specifically targeted α-smooth muscle actin positive liver myofibroblasts within scar regions of the liver in vivo and did not co-localise with liver monocytes/macrophages. Injection of free gliotoxin stimulated a two-fold increase in non-parenchymal cell apoptosis and depleted liver myofibroblasts by 30% and monocytes/macrophages by 50% but had no effect on fibrosis severity in the sustained injury model employed. In contrast, C1-3-targeted gliotoxin stimulated a five-fold increase in non-parenchymal cell apoptosis, depleted liver myofibroblasts by 60%, did not affect the number of monocytes/macrophages and significantly reduced fibrosis severity. Fibrosis reduction was associated with increased metalloproteinase-13 levels. Conclusions: These data demonstrate that specific targeting of liver myofibroblast apoptosis is the most effective anti-fibrogenic therapy, supporting a role for liver monocytes and/or macrophages in the promotion of liver fibrosis reduction.

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1. Introduction

Liver fibrosis is a common outcome in patients with chronic liver damage of any cause [1]. It is associated with the activity of liver-resident fibroblasts, derived from the trans-differentiation of hepatic stellate cells (HSCs) to myofibroblast-like cells or the proliferation of portal fibroblasts [1]. The fibrogenic response is a component of the inflammation that occurs in response to hepatocyte necrosis, with liver myofibroblasts likely functioning to re-model the extracellular matrix prior to tissue regeneration [1].

Most clinical liver disease is associated with chronic liver injuries and excessive inflammation. [1]. Liver myofibroblasts generate scarring within the tissue that ultimately leads to cirrhosis. Under these conditions, the liver is unable to regenerate and the only treatment available at present is liver transplantation [1]. This laboratory [2,3] and others [4-8] have shown that a stimulation of pro-fibrogenic liver myofibroblast apoptosis reduces scarring in recovery animal models of liver fibrosis. These experiments suggest that reductions in the numbers of liver myofibroblasts in a fibrotic liver may be an effective therapeutic approach to chronic liver disease. However, it is now clear that a complex relationship exists between various liver cell types in the diseased liver. The initial switch that triggers a fibrogenic response in liver myofibroblasts has not been unequivocally identified but there is good evidence to suspect that liver-resident macrophages (Kupffer cells) are its (major) source [1]. Further, many of the pro-inflammatory cytokines produced by Kupffer cells promote liver myofibroblast fibrogenic activity, most notably interleukin 6, TNF-α and TGF-β [9-13]. The prominent role of Kupffer cells in fibrogenesis was elegantly demonstrated by Duffield et al in which fibrosis was significantly reduced in a mouse model of disease when Kupffer cells were depleted from the liver [14]. Surprisingly
however, Kupffer depletion also inhibited the normal reversal of fibrosis that can occur prior to cirrhosis when liver damage was halted [14]. Therefore, an effective anti-fibrotic therapy reliant on the stimulation of apoptosis may likely need to be specifically targeted to liver myofibroblasts for optimum efficacy.

Synaptophysin is a membrane protein present in a restricted number of cell types such as neural cells. It is thought to function in the release and/or uptake of neurotransmitters [15]. In the liver, synaptophysin expression is reported to be restricted to HSC-derived myofibroblasts (myofibroblasts express a number of genes associated with neural tissue) [14-19]. Synaptophysin's external cellular location and cycling to intracellular location(s) make it a potential site for targeting liver myofibroblasts with therapeutics. Recombinant human monoclonal single chain antibodies (scAbs) were therefore generated to a conserved peptide sequence present in an extracellular domain of synaptophysin [20].

In this study we show that the C1-3 scAb does not cross the blood brain barrier and targets liver myofibroblasts in vivo in a mouse model of liver fibrosis. In addition, gliotoxin - a compound shown to stimulate apoptotic-resistant fibrogenic human HSC apoptosis in vitro [37], when chemically conjugated to C1-3, significantly reduced the numbers of liver myofibroblasts present in the fibrotic disease model without affecting Kupffer cell numbers. The effects of the C1-3-gliotoxin conjugate were compared to the effects of free gliotoxin and a control scAb-gliotoxin conjugate (CSBD9, a scAb raised to microcystin [21], which should not bind to the surface of liver cells).
2. Materials and methods

2.1. Recombinant scAb expression and conjugation.

The polyhistidine tagged recombinant scAbs - C1-3 (to synaptophysin) and CSBD9 (to microcystin-LR [21] and used as a control in these studies) were expressed in *E. coli* and purified by nickel chromatography and gel filtration essentially as previously described [20,21]. Endotoxin was removed from preparations using Q Maxi H columns (Sartorius Vivascience, Epsom, UK) and all preparations were tested (< 0.2 EU/ml) by Lonza Biologics (Slough, UK) prior to use *in vivo*. Purified scAbs were labelled with FITC as previously outlined [20]. Gliotoxin was conjugated to scAbs essentially as outlined [22] – see also supplementary Fig. 1. Protein conjugates were analysed by an Applied Biosystems Voyager DE-STR-MALDI-TOF mass spectrometer (Aberdeen Proteomics). Gliotoxin conjugation was further confirmed by Western blotting as outlined [23] using an antibody to gliotoxin supplied by Dr Sean Doyle (National University of Ireland, Maynooth, Ireland). ScAb conjugates contained < 0.2 % gliotoxin in its free form (supplementary Fig. 2).

2.2 In vitro functional tests of recombinant scAb conjugate activity.

Antigen binding ELISA with peptide 2 (YPFRLHQVYFDAPSC sequence to which the C1-3 scAb binds) and peptide 1 (ATDPENIIKEMPMC, an unrelated
sequence also present within synaptophysin) as a control were performed using BSA-peptide conjugates as outlined [20].

2.3 BIAcore.

BSA conjugates with peptides 1 or peptide 2 were covalently immobilised to a CM5 sensor chip (GE Healthcare, Amersham, UK) and scAb binding kinetics determined as outlined [24].

2.4 HSC isolation and culture.

Male adult mice (C57Bl6) were used in all studies and were purchased from Harlan. Mouse HSCs were isolated from mechanically disrupted whole liver as outlined [26] and cultured as described [2,25]. The cells were sub-cultured by trypsin digestion and were used between passages 2 and 4.

2.5 Western blotting.

Western blotting was performed as outlined [26] using HRP-conjugated secondary antibodies and detection with ECL reagent (Amersham) and autoradiography. Goat anti-human Cκ light chain was used to detect C1-3 scAb and was purchased from the Sigma Chemical Co. Poole, UK. Mouse monoclonal
antibodies to $\alpha$-smooth muscle actin and $\beta$-actin were from Sigma and Chemicon (Chandlers Ford, UK) respectively.

2.6 *CCl₄ in vivo model of liver fibrosis and scAb treatment.*

Mice were administered CCl₄ mixed 1:1 (v/v) with olive oil - 2ml/kg body weight by *i.p.* injection - twice weekly for 8 weeks to generate liver fibrosis. Control animals were administered olive oil alone (1ml/kg body weight). Free gliotoxin was administered in DMSO at 60 µg / 100 g body weight (0.5 mg gliotoxin/ml), control animals received DMSO (120 µl / 100g body weight) alone. All scAbs and scAb conjugates were prepared in phosphate buffered saline (PBS 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate pH 7.4) and administered at 2 mg scAb / 100 g body weight (from a 1 mg/ml PBS stock by *i.p.* injection). As scAb conjugates, mice received the equivalent of 60 µg gliotoxin / 100 g body weight. At various timepoints, mice were anaesthetised using isoflurane and blood taken by cardiac puncture. All animals were then killed by cervical dislocation and samples of various tissues snap-frozen in dry ice or fixed in 10 % formalin in PBS. Blood was allowed to clot prior to centrifugation to obtain serum for analyses.

2.7 *Tissue analysis.*

Serum ALT levels, sirius red staining and $\alpha$-smooth muscle actin immunostaining were performed as previously outlined [2]. The mouse monoclonal $\alpha$-smooth muscle actin antibody was purchased from Sigma (Poole, UK), and sections
then developed using the mouse on mouse Vectastain system (Vector Labs, Peterborough, UK). Immunostaining tissue for C1-3 scAb was undertaken using HRP-conjugated goat anti-human Cκ light chain antibody followed by DAB colour development. Anti-F4/80 (for monocyctic and Kupffer cells in formalin fixed tissue) and metalloproteinase-13 (MMP-13) antibodies were purchased from Abcam. Frozen tissue was sectioned at a thickness of 6 µm in O.C.T. embedding compound. In co-localisation studies, sections were further incubated with Alexa Fluor 594-conjugated antibodies (either the anti-α-smooth muscle actin above or mouse anti- ED-1, note anti-ED-1 was more effective than anti-F4/80 in frozen sections). Sections were then DAPI mounted and examined using a UV microscope.

3. Results

ScAb conjugation does not significantly affect antigen affinity and conjugate activity in vitro.

C1-3 and CSBD9 ScAbs were conjugated with FITC or gliotoxin. Figure 1A demonstrates that the mean molecular weight of the C1-3 scAb after conjugation with gliotoxin increased by an average of 1.5 kDa indicating that on average 3.5 molecules of gliotoxin had been conjugated to the C1-3 scAb protein (see supplementary Figure 1). Similar results were observed with the CSDB9 scAb (data not shown). FITC conjugation generally resulted in the conjugation of 4-5 molecules of fluorescein per molecule of protein (data not shown). Western blotting using an antibody raised to gliotoxin confirmed that gliotoxin had been conjugated to proteins (Figure 1B) and additional analysis confirmed that free gliotoxin did not remain within the
preparations – see supplementary Figure 2). Figures 1C and D demonstrate that despite conjugation with gliotoxin or FITC, C1-3 scAb retained its affinity for peptide 2 (the sequence originally used during panning for phage expressing antibody for synaptophysin [20]) with no affinity for peptide 1 or the BSA carrier protein. CSBD9 scAb and its conjugates did not bind to peptide 1 or 2 sequences (Figure 1D) although affinity for its antigen - microcystin – was not affected (Figure 1E). Figure 1F demonstrates that the gliotoxin conjugate retained almost identical kinetics for peptide 2 binding, with C1-3 and C1-3-GT showing $<10^{-7}$ M affinity (Table 1).

The isolation and culture of HSCs on plastic in serum-containing medium results in trans-differentiation to a myofibroblast-like cell with phenotypically similar characteristics to the cells present in fibrotic liver [1]. Figure 2A demonstrates that the expression of the C1-3 scAb antigen was markedly increased in human HSC-derived myofibroblasts and was present in the human stellate-like cell line LX-2 [24]. The antigen was also present in mouse myofibroblasts from C57Bl6 mice. C1-3-FITC scAb readily interacted with a surface antigen on mouse myofibroblasts under native conditions (Figure 2B). Confocal microscopy shows that the C1-3 scAb bound to the plasma membrane of cells but also that there was intracellular binding of a lower intensity (Figure 2B). Gliotoxin stimulates a rapid apoptosis of rat and human HSC-derived myofibroblasts [2,23]. Addition of C1-3-GT or free gliotoxin to mouse HSC-derived myofibroblasts in vitro resulted in cell death whereas the C1-3 scAb protein alone was not toxic (Figure 2C and 2D). CSBD9 and CSBD9-GT were not toxic to mouse myofibroblasts (Figure 2D) indicating that C1-3-GT-derived gliotoxin uptake was dependent on C1-3 interaction with its antigen. The abilities of C1-3 scAb to interact with its antigen and gliotoxin to stimulate cell death were therefore not lost by their conjugation.
The C1-3 scAb targets mouse HSCs in vivo.

To establish whether the C1-3 scAb is able to target liver myofibroblast in vivo, mice were administered CCl₄ for 8 weeks to generate fibrosis (Figure 3A). A comparison of indices of liver damage and fibrosis indicates that animals administered CCl₄ had significant liver damage (elevated serum ALT) and liver myofibroblast proliferation, see Figure 3B. Figure 3C shows that C1-3 scAb entered the systemic circulation within 20 minutes of i.p. administration and had a half-life of approximately 2 hours. Analyses of tissue homogenates (liver, kidney, brain, skeletal muscle and adipose tissue) indicates that C1-3 scAb was detectable in kidney transiently (at 20 minutes) and for between 2 – 6 hours in the liver (Figure 3C). All other tissues were negative for C1-3 at all time points (data not shown). Immunohistochemical examination of tissues with an antibody to the Cκ light chain tag present in the scAb indicates that C1-3 was present in the livers of mice with fibrosis but not control mice (Figure 3D). C1-3 was not detected in any other tissue by this method, including the kidney and brain (Figure 3E). The appearance of C1-3 in kidney extracts may therefore have been due to free scAb within blood that contaminated tissue homogenates to varying degrees. C1-3 scAb targeted cells within the scar of fibrotic livers (Figure 3D) and was not present within the parenchyma. Cell counts for C1-3-positive cells in randomly selected central veins indicate that the number of positive cells increased after administration of scAb to a peak at 6 hours and subsequently fell to a lower but significant level by 24 hours (Figure 3F).

Minimal evidence for C1-3 immunoreactivity was observed in fibrotic liver tissue cut from archived standard formalin-fixed/paraffin-embedded tissue (data not shown), suggesting that the antigenic site for the C1-3 scAb is destroyed by formalin
fixation. In contrast, C1-3 scAb readily bound to frozen sections of (native) fibrotic liver tissue (not shown). Staining liver sections from mice that had previously been injected with C1-3-FITC scAb indicates that the C1-3-FITC bound to cells within the fibrotic liver but not control liver (Figure 4A). Figure 4B confirms that the C1-3-FITC antibody co-localised with α-sma positive cells (Figure 4B) but not ED-1 positive monocytes and/or macrophages (Figure 4C). These data suggest therefore that the C1-3 scAb was bound specifically to liver myofibroblasts in vivo.

**Gliotoxin-dependent reversal of fibrosis in vivo is significantly enhanced through its conjugation to the C1-3 scAbs.**

Previous work has demonstrated that the fungal metabolite gliotoxin is a potent stimulator of myofibroblast apoptosis in vitro through its dual action of an inhibition of NF-κB and promotor of mitochondrial membrane permeability [2,23]. Gliotoxin administration reversed fibrosis in vivo in recovery rat models [2,4]. However, gliotoxin also stimulates the apoptosis/necrosis of Kupffer cells [27,28] which have been shown to be promote more effective fibrosis reversal [14]. Further, under inflammatory conditions, hepatocytes may be more susceptible to cell death if NF-κB is inhibited [2,23].

We hypothesised that fibrosis reversal with gliotoxin would be more effective if myofibroblast apoptosis was specifically targeted. To test this hypothesis, free gliotoxin was administered to mice with liver fibrosis (Figure 5A) and its effects within the liver compared to an equivalent dose of gliotoxin as a conjugate with C1-3 (C1-3-GT) or the control scAb CSBD9 (CSBD9-GT). A pilot experiment showed that free gliotoxin was toxic to CCl₄-treated mice at 3 mg/kg body weight (the dose which most effectively reversed fibrosis in a rat recovery model [2]). Accordingly, all
mice received a reduced 0.6 mg gliotoxin/kg body weight as either a free or a scAb conjugate form.

Figure 5B demonstrates that free gliotoxin increased by two-fold non parenchymal cell apoptosis and significantly reduced by 30% the number of α-sma positive cells in the liver, essentially as observed previously in a rat model of liver fibrosis recovery [2]. Figure 5C shows that C1-3-GT was more effective in that non parenchymal cell apoptosis was increased 5-fold and the number of myofibroblasts present in the liver was significantly reduced by 60%. The lack of effect of the CSBD9-GT scAb confirms that the C1-3 scAb specifically directs gliotoxin to myofibroblasts in vivo. Figure 6A and B confirm that free gliotoxin treatment significantly reduced the number of Kupffer cells and MMP-13 positive cells present in the liver, and fibrosis severity was not markedly affected by gliotoxin in the sustained model employed here. In contrast, C1-3-GT had no effects on the number of Kupffer cells present in the liver (Figure 6C), the levels of MMP-13 were maintained (Figure 6D) and fibrosis severity was significantly reduced (Figure 6D). Taken together, these data therefore suggest that the C1-3 scAb directs gliotoxin away from Kupffer cells, promotes increased myofibroblast apoptosis and reduces fibrosis.
4. Discussion

There are currently no treatments indicated for liver fibrosis [29]. In animal models of reversible fibrosis where the liver damaging agent is withdrawn (i.e. recovery), fibrosis resolution is accompanied by the apoptosis of liver myofibroblasts [30]. Previous work demonstrated – with free gliotoxin – that fibrosis resolution in rats was accelerated by stimulating the apoptosis of myofibroblasts [2,4]. In these studies, there was evidence in liver sections for an increase in the apoptosis of other cell types in response to gliotoxin although proportionately significantly less extensive than observed with myofibroblasts [2]. Since the presence of Kupffer cells in the liver promotes the accumulation of fibrosis and the resolution in recovery [14], a potential unwanted side-effect of an anti-fibrogenic that mediates myofibroblast apoptosis, could be the stimulation of Kupffer cell apoptosis in recovery. Recent data indicated that free gliotoxin stimulates the apoptosis and necrosis of Kupffer cells \textit{in vitro} and \textit{in vivo} [28,31]. Targeted myofibroblast apoptosis and its effects on liver fibrosis were therefore investigated.

The selectivity of our targeting scAb for liver myofibroblasts was demonstrated using an \textit{in vivo} mouse model in which fibrogenesis was ongoing (treatment with scAbs or free gliotoxin was followed by further liver damage with CCl\textsubscript{4}). The effects of free gliotoxin and targeted gliotoxin on fibrosis were tested. In agreement with others [28,31], free gliotoxin significantly reduced the number of Kupffer cells present within the liver. However, C1-3-targeted gliotoxin had no effects on the numbers of Kupffer cells. The targeted apoptosis of myofibroblasts resulted in a more effective reduction in the numbers of \(\alpha\)-sma positive liver myofibroblasts and fibrosis severity. These data therefore support the concept that
myofibroblast reduction reverses and reduces severity in animal models of fibrosis. It has been postulated that Kupffer cell-derived MMP13 expression makes a major contribution to fibrosis catabolism [32] and this is supported by our experiments. The specific removal of myofibroblasts, whilst retaining the presence of Kupffer cells, may account for the enhanced reduction of fibrosis in C1-3-GT treated mice compared to free gliotoxin treated mice.

Whilst this paper provides data in support of the hypothesis that retaining Kupffer cells whilst reducing myofibroblasts is an effective approach to reducing fibrosis severity, there remain some questions that need to be addressed with regard to myofibroblast apoptosis and anti-fibrogenic therapies. These include the need to test the theory in additional models of liver disease since the population of myofibroblasts that proliferate in response to various disease aetiologies may be different [33]. Further, efficacy in models of severe liver disease needs to be tested. Myofibroblast apoptosis results in a rapid reduction in fibrosis in the current models employed, suggesting that scar turnover may be continuous and relatively rapid. Cirrhosis (or “irreversible” fibrosis) in which excessive scarring is considered un-degradable, may yet resolve if in reality, scar is directly associated with the numbers of myofibroblasts and their intrinsic fibrogenic potential. The ability of C1-3-GT to reverse fibrosis in models in which fibrosis is believed to not fully resolve [34] needs to be examined.

This laboratory originally used gliotoxin as a model compound for examining the role of myofibroblast apoptosis as a therapy for fibrosis because it is potent and rapidly effective at killing myofibroblasts in vitro and in vivo [2,23]. Gliotoxin is an NF-κB inhibitor [35,36] but interestingly, was the only effective compound - in a range of NF-κB inhibitors – to stimulate the apoptosis of highly fibrogenic (passaged) human myofibroblasts in vitro [37]. It is likely that its efficacy is associated with both
an inhibition of NF-κB and a direct interaction with mitochondrial permeability [23].

In view of the potential resistance of human myofibroblasts to apoptosis in cirrhosis, effective targeting of gliotoxin to myofibroblasts using the C1-3 scAb or similar targeting agent should be considered as a potential treatment for severe fibrosis/cirrhosis in patients followed by milder anti-fibrogenics which inhibit myofibroblast proliferation and/or fibrogenicity [25].

5. Acknowledgements

AD and KW are supported by studentships from the BBSRC.

6. References.


7. Figure legends.

**Figure 1. ScAb conjugation and antigen selectivity.**

A, MALDI-TOF for purified C1-3 scAb (left panel) and C1-3-GT (right panel). B, Detection of covalently-bound gliotoxin in C1-3 or bovine serum albumin (BSA) prior to and after the conjugation procedure outlined in the Methods section. C1-3-SATA and BSA-SATA, proteins activated with conjugation agent prior to addition of activated gliotoxin, demonstrating selectivity of the antibody for gliotoxin (5 µg/lane). Predicted monomer protein-gliotoxin conjugates are indicated by arrows (additional higher molecular weight bands with C1-3 are likely to be multimers). C, typical antigen binding ELISA (mean ± standard deviation 3 separate determinations) demonstrating selectivity of C1-3 scAb for BSA-peptide 2 versus BSA-peptide 1 and employed in D to show that scAb conjugation does not affect scAb binding to BSA-peptide 2 for C1-3 or the control scAb CSBD9 (100 µg scAb/ml) or E, CSBD9 scAb binding to its microcystin antigen (100 µg scAb/ml). F, typical BIAcore association and dissociation data for C1-3 and C1-3-GT scAbs employed in this study (see also Table 1).

**Figure 2. Conjugation does not affect scAb binding to human and C57Bl6 mouse myofibroblasts or drug activity in vitro.**

A, Western blot for the presence of C1-3 antigen using the C1-3 scAb, α-smooth muscle actin and total actin. Each lane contains 10 µg cell protein/lane, data typical of 3 separate experiments. B, Left panels - immunofluorescence with unlabelled C1-3 or C1-3-FITC in mouse myofibroblasts. The indicated scAbs (10 µg/ml) were incubated with cells in culture media for 1 hour. The cells were then washed several times with HEPES/HBSS [23] prior to examination. All views taken under the same
lighting and exposure conditions. Right panel, mouse myofibroblasts sub-cultured onto glass coverslips were washed in PBS, fixed (10 minutes in ice-cooled methanol followed by 10 minutes in 2% w/v formaldehyde / 0.2% w/v glutaraldehyde in PBS, pH 7.4) washed in PBS and then incubated with C1-3-FITC for 1 hour at rtp. The cells were then washed in PBS and mounted in Vectashield containing propidium iodide prior to analysis by confocal microscopy. Green, C1-3-FITC; red, nucleus DNA. Data typical of 5 separate experiments. C, timecourse for the effects of free gliotoxin or C1-3-GT on mouse myofibroblast sub-stratum adherence in vitro. Culture-activated cells were sub-cultured into 24 well plates in 300 µl medium and treated with either free gliotoxin added from a 1000-fold molar concentrated stock in DMSO vehicle (total 450 pmoles/well); 4.5 µg C1-3 / well or 4.5 µg C1-3-GT scAb (to give approx. 450 pmoles gliotoxin /well for conjugated scAb). Data are the mean and standard deviation of 3 separate preparations. D, mouse myofibroblast attachment after 12 hours after incubation with the indicated scAb: GT, gliotoxin. Data are the mean and standard deviation of 10 randomly selected fields from the same experiment, typical of 3 separate experiments. Significantly different (two tailed) using the Students T test from *DMSO control or ¶C1-3 scAb (P > 95%).

Figure 3. The CCl₄ model of liver fibrosis and the C1-3 targeting of liver scars after injection. A, schematic diagram of study 1, to determine whether C1-3-FITC binds to myofibroblasts in the fibrotic liver. B, evidence for liver damage and myofibroblast proliferation in mice administered CCl₄ – left, increases in serum alanine aminotransferase (ALT) activity, mean and standard deviation from 3 animals per group; right, α-smooth muscle positive myofibroblasts, data typical of 3 animals 24 hours after injection with C1-3-FITC or PBS. C, Western blot for C1-3-FITC
scAb in serum prepared via cardiac bleed (10 µg/lane), liver (10 µg/lane) and kidney (10 µg/lane) at the time points indicated. C1-3-FITC, purified standard (1 µg/lane). Tissue sections from control or fibrotic liver (6 h post injection), D, or brain and kidney (6 h post injection), E, immunostained for C1-3-FITC via anti-human Cκ light chain tag. Right panels are magnification of box outlined on corresponding left panel. Positive staining is brown. F, blind scoring (10 fields/animal and 3 animals / time point) of C1-3-FITC positive cells in randomly selected centrlobular regions from fibrotic mice at the indicated time after administration of C1-3-FITC. All data in D-E typical of at least 3 animals per time point.

Figure 4. The C1-3-FITC scAb targets liver myofibroblasts in fibrotic mouse liver.
A. control and fibrotic liver sections from mice 6 hours after injection with C1-3 or C1-3-FITC scAbs. Liver was snap-frozen and sections were mounted with DAPI Vectastain (stains nuclei blue) followed by analysis by fluorescence microscopy. Fibrotic liver sections from mice 6 hours after injection with C1-3-FITC scAb then incubated with anti-α-sma-AF-594 antibody (red) (B) or anti-ED-1-AF-594 antibody (red) (C), coverslipped with DAPI-containing Vectashield and analysed by confocal microscopy. Typical results shown.

Figure 5. C1-3-GT scAb targets myofibroblasts and has greater efficacy over free gliotoxin to stimulate myofibroblast apoptosis in mouse liver in vivo.
A, schematic diagram of study 2, to determine whether free gliotoxin or scAb-GT conjugate administration modulates liver fibrosis. B, Representative immunostaining for α-smooth muscle actin in liver sections (left) from mice treated with gliotoxin...
dissolved in DMSO (GT) or DMSO alone. Quantitative analysis for non parenchymal active caspase 3 (typical view, inset) and α-sma positive cells - *Significantly different (two tailed) versus DMSO. C, Representative immunostaining for α-smooth muscle actin in liver sections (upper panel) from mice treated with scAb and scAb-conjugates (dissolved in PBS) or PBS alone. Quantitative analysis for non parenchymal active caspase 3 and α-sma positive cells. Significantly different (two tailed) versus $^5$PBS or $^6$C1-3 treated groups. Note, administration of various treatments did not affect the levels of liver damage as determined histologically (data not included) or from serum levels of ALT (supplementary Figure 3). All data are the mean and standard deviation from at least 5 animals / treatment group with typical views presented and data tested for statistical significance using the Students T test (P > 95%).

Figure 6. Conjugation of gliotoxin to C1-3 scAb abrogates the loss of Kupffer cells and enhances reductions in liver fibrosis.

A, Typical liver sections from animals treated with free gliotoxin dissolved in DMSO (GT) or DMSO only and immunostained for F4/80 for the detection of Kupffer cells (brown) – right upper panel is a high magnification view (K, Kupffer; H, hepatocyte). Bargraph, quantitative analysis for F4/80 positive cells B, Liver sections immunostained for MMP-13 and quantitation of the number of MMP-13 positive cells (upper panels) or stained with sirius red (lower panels). Right lower, quantitative analysis for fibrosis severity in sirius red stained sections [26]. C, Typical liver sections from animals treated with scAb and scAb-conjugates (dissolved in PBS) or PBS alone and immunostained for F4/80 for the detection of Kupffer cells (brown). Bargraph, quantitative analysis for F4/80 positive cells. D, Liver sections immunostained for MMP-13 (upper panels) or stained with sirius red (lower left
panels). Right lower, quantitative analysis for fibrosis severity in sirius red stained sections [26]. Quantitation were made from 10 randomly selected fields per animal with at least 5 animals per group. *Significantly different (two tailed) number of positive cells versus DMSO. The mean and standard deviation sirius red score for each group is indicated with a horizontal and vertical line respectively. *Significantly different (two tailed) severity versus C1-3 treated group using the Students T test (P > 95%).
Figures

A

B

C

D

E

F

Figure 1
Figure 2
Figure 3
Figure 3 contd
Figure 4
Figure 5 part 1
Figure 5 part 2
Figure 6

A

[DMSO image]

[GT image]

[Graph showing mean number of apoptotic nuclei per field of view for DMSO and GT treatments]