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Jessica Gewiese-Rabsch, Claudia Drucker, Sven Malchow, Jürgen Scheller, Stefan Rose-John. Role of IL-6 trans-signaling in CCl induced liver damage. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2010, 1802 (11), pp.1054. 10.1016/j.bbadis.2010.07.023 . hal-00623297

HAL Id: hal-00623297

<https://hal.science/hal-00623297>

Submitted on 14 Sep 2011

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Accepted Manuscript

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PII: S0925-4439(10)00160-2
DOI: doi: [10.1016/j.bbadis.2010.07.023](https://doi.org/10.1016/j.bbadis.2010.07.023)
Reference: BBADIS 63148

To appear in: *BBA - Molecular Basis of Disease*

Received date: 18 May 2010
Revised date: 20 July 2010
Accepted date: 29 July 2010



Please cite this article as: Jessica Gewiese-Rabsch, Claudia Drucker, Sven Malchow, Jürgen Scheller, Stefan Rose-John, Role of IL-6 trans-signaling in CCl₄ induced liver damage, *BBA - Molecular Basis of Disease* (2010), doi: [10.1016/j.bbadis.2010.07.023](https://doi.org/10.1016/j.bbadis.2010.07.023)

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Role of IL-6 trans-signaling in CCl₄ induced liver damage

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Keywords: Interleukin-6, carbon tetrachloride, liver damage, regeneration, IL-6 trans-signaling

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ABSTRACT

Interleukin-6 (IL-6) plays an important role in liver regeneration and protection against liver damage. In addition to IL-6 classic signaling via membrane bound receptor (mIL-6R), IL-6 signaling can also be mediated by soluble IL-6R (sIL-6R) thereby activating cells that do not express membrane bound IL-6R. This process has been named trans-signaling. IL-6 trans-signaling has been demonstrated to operate during liver regeneration. We have developed methods to specifically block or mimic IL-6 trans-signaling. A soluble gp130 protein (sgp130Fc) exclusively inhibits IL-6 trans-signaling whereas an IL-6/sIL-6R fusion protein (Hyper-IL-6) mimics IL-6 trans-signaling. Using these tools we investigate the role of IL-6 trans-signaling in CCl₄ induced liver damage. Blockade of IL-6 trans-signaling during CCl₄ induced liver damage led to higher liver damage, although induction of Cyp4502E1 and thus bioactivation of CCl₄ was unchanged. Depletion of neutrophils resulted in reduced liver transaminase levels irrespective of IL-6 trans-signaling blockade. Furthermore, IL-6 trans-signaling was important for refilling of hepatocyte glycogen stores, which were depleted 24 h after CCl₄ treatment. We conclude that IL-6 trans-signaling via the soluble IL-6R is important for the physiologic response of the liver to CCl₄ induced chemical damage.

189 words

1. INTRODUCTION

Interleukin 6 (IL-6) is an inflammatory cytokine with additional functions outside the immune system. Two different pathways have been described for IL-6. In the classical IL-6 pathway, IL-6 binds to its specific Interleukin-6-receptor (IL-6R) and the IL-6/IL-6R complex binds to the transducing receptor glycoprotein 130 (gp130) leading to homodimerization and subsequent activation of the STAT3 pathway. IL-6R is only expressed on some cells, mainly on hepatocytes and leukocytes. However, IL-6 can also signal via soluble IL-6R (sIL-6R) that is generated via proteolytic cleavage or, in humans also via alternative splicing. This alternative pathway, which enlarges the range of IL-6 target cells, is called IL-6 trans-signaling .

Using transgenic mice we have shown that the IL-6/sIL-6R complex (i.e. activation of IL-6 trans-signaling) but not IL-6 alone was capable to induce hepatocyte proliferation even in the absence of liver damage. Using gene deficient mice it was shown that IL-6 plays an important role in liver regeneration . We developed Hyper-IL-6, a fusion protein of IL-6 bound to the sIL-6R which mimics IL-6 trans-signaling . *In vivo*, Hyper-IL-6 but not IL-6 alone, induced hepatocyte proliferation after hepatectomy or D-galactosamine induced liver damage, demonstrating the potential of IL-6 trans-signaling to accelerate liver regeneration .

IL-6 trans-signaling can specifically be blocked by a soluble gp130Fc fusion protein (sgp130Fc) without affecting IL-6 signaling via the membrane bound IL-6R . This sgp130Fc protein was used to demonstrate that endogenous IL-6 trans-signaling was important for chronic inflammation states and colon cancer . Recently, we could demonstrate that sgp130Fc reduced glycogen consumption in the liver of animals treated with D-galactosamine .

CCl_4 is a hepatotoxin that causes direct hepatocyte damage by altering the permeability of cellular, lysosomal and mitochondrial membranes. Furthermore, CCl_4 is metabolized by the cytochrome P450-dependent monooxygenase Cyp450 2E1 forming the reactive CCl_3^* and Cl_3COO^* radicals, which can covalently bind to proteins, lipids and nucleic acids and thus induce liver damage and initiate lipid peroxidation. Recently, it has been shown that CCl_4 not only causes primary liver necrosis, but also hepatocyte apoptosis.

IL-6 deficient mice were shown to be more sensitive to CCl_4 damage. Interestingly, this effect could not be compensated by recombinant IL-6 but only by Hyper-IL-6 indicating a role of IL-6 trans-signaling in the response to CCl_4 liver damage.

In the present study we focus on the role of IL-6 trans-signaling in response to liver injury caused by CCl_4 . By blocking endogenous IL-6 trans-signaling we demonstrate for the first time the importance of the sIL-6R in response to chemically induced liver damage.

2. MATERIALS AND METHODS

2.1 Animals and CCl₄ treatment.

Male C57Bl/6N mice were kept at a 12-hour light-dark cycle under standard conditions and provided with food and water *ad libitum*. Sgp130 transgenic animals were generated and treated identically to C57Bl/6N for flow cytometric analysis of neutrophils. For all experiments 4 – 6 mice per group were used. Liver damage was induced by intraperitoneal injection (i.p.) of CCl₄ (Sigma, Deisenhofen, Germany) dissolved in rape oil (20 % v/v) immediately before treatment and applied as one dose of 3 ml/kg body weight. All experiments were performed according to the German guidelines for animal care and protection (V 31272241.121-3 (41-3/06)).

2.2 Treatment and quantification of sgp130Fc levels via Enzyme-linked immunoabsorbent assays.

Mice were treated i.p. with 250 µg sgp130Fc 18 h prior to CCl₄ treatment. sgp130Fc levels were measured via Enzyme-linked immunoabsorbent assays (ELISA) in the serum of the mice as described using a human gp130 Elisa Kit (DuoSet human gp130 ELISA Kit, R&D Systems, Wiesbaden, Germany) according to the manufacturers' instructions. Serum was diluted 1:2,000 in 1% BSA/PBS and measured in duplicates. Recombinant gp130 was used as standard.

2.3 Induction of IL-6 trans-signaling with Hyper-IL-6.

Mice were treated with 4 µg Hyper IL-6 i.p. 18 h prior to CCl₄ treatment.

2.4 Neutrophil depletion. As described previously, neutrophils were depleted using a purified rat anti-mouseLy6G/Ly6C monoclonal antibody (mAb) (BD Bioscience, Heidelberg,

Germany). Mice were injected with 100 µg mAb i.p. 18 h prior to CCl₄. Depletion was controlled with stainings of neutrophils on paraffin tissue sections as described below.

2.5 Flow cytometric analysis.

20 µl of whole blood samples were used for FACS analysis, whereby the mAbs Ly6GC (BD Biosciences, Heidelberg, Germany) and CD11b (BD Biosciences, Heidelberg, Germany) were used to count infiltrating neutrophils. Immediately, blood was transferred into 100 µl FACS EDTA buffer (2 mM EDTA in PBS) to prevent clotting and inverted briefly. For each staining 100 µl of blood/ EDTA buffer mixture was transferred into a well of a 96-well-plate. To block Fc-receptors on, the suspension was incubated with mouse Fc Block CD 16/32 mAb (BD Biosciences). The cells were subsequently treated with the fluorescence coupled mAbs for 30 min. Thereafter 96-well-plate was centrifuged at 1,500 rpm for 5 min at 4°C, supernatant was discarded and pellet was immediately resuspended in 100 µl 1 x FACS Lysing solution (BD Biosciences). Wells were washed twice with 100 µl FACS buffer (PBS, 1% BSA, 1 g/L N3Na) and resuspended in 200 µL PBS and analyzed by FACS (FACSCanto; Becton Dickinson, Heidelberg, Germany). In general, data were acquired from 10 000 gated events per sample.

2.6 Serum alanine aminotransferase, serum aspartate aminotransferase, potassium and uric acid measurements.

Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), potassium and uric acid were determined using a Reflotron analyzer (Roche Diagnostics, Basel, Switzerland) and Reflotron test strips. These parameters were determined in diluted blood sera.

2.7 Soluble IL-6R serum concentrations.

sIL-6R levels were measured via ELISA using a murine IL-6R ELISA Kit (DuoSet murine IL-6 ELISA Kit, AF1830, R&D Systems, Wiesbaden, Germany) according to the manufacturers instructions. Serum was diluted 1:10 in 1% BSA/PBS and measured in duplicates. Recombinant mouse IL-6R was used as standard.

2.8 IL-6 serum concentrations.

IL-6 levels were measured via enzyme-linked immunoabsorbent assays using a murine IL-6 ELISA Kit (DuoSet murine IL-6 ELISA Kit, DY406, R&D Systems, Wiesbaden, Germany) abiding to the manufacturers instructions. Serum was diluted 1:10 in 1% BSA/PBS and measured in duplicates. Recombinant mouse IL-6 was used as standard.

2.9 Protein preparation and Western blotting.

Livers were homogenized in lysis buffer (500 mM NaCl, 50 mM Tris, pH7.4; 0.1% SDS, 1% NP-40) and Western blots were performed as described previously . 40 µg of protein in 5x Laemmli buffer was applied onto SDS-polyacrylamide gels, electrophoretically separated, and transferred onto PVDF-membranes. After blocking in Tris buffered saline (TBS, 0.3 M NaCl, 0.05 M Tris) with 0.05 % Tween and 5 % skim milk, membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted 1:1,000 in blocking solution. The following mAbs were used: pSTAT3, STAT3, β-Actin (Cell Signaling, Boston, USA) and Cyp2E1 (Abcam, Cambridge, UK). Thereafter, membranes were washed and incubated with horseradish-coupled secondary antibodies (Amersham Bioscience, Buckinghamshire, U.K.) at a dilution of 1:5,000. Signals were visualized using enhanced chemiluminescence detection system (ECLplus Amersham-Biosciences, Buckinghamshire, U.K.). The membranes were exposed to the Image reader LAS-100 Pro system (Fujifilm, Düsseldorf, Germany) or Fuji Films (Amersham-Biosciences, Buckinghamshire, U.K.).

2.10 Tissue processing, immunohistochemistry, TUNEL staining.

Liver tissue was fixed in 4% formaline, processed and immunostained. Staining for Apoptosis was carried out using a Peroxidase in Situ Apoptosis Detection Kit (Chemicon International, Billerica, USA). The signal was developed with DAB Substrate (3,3' Diaminobenzidine, Dako, Glostrup, Denmark) and samples were counterstained with methylgreen (Serva, Heidelberg, Germany).

2.11 HE and DAPI staining.

Tissue sections were shortly incubated in Gill3 Hematoxylin (Thermo Scientific, Cheshire, UK), differentiated in 0.5% acetic acid, rinsed in tap water and stained with Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany). Necrotic areas were quantified using ImageJ-Software. Necrotic areas were calculated from 30 random high-powered fields of three mice and given in percent of total area. Tissue sections were stained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) (Sigma, Deisenhofen, Germany) diluted 1:1,000 in PBS for 10 min.

2.12 Periodic Acidic Schiff (PAS) staining.

Glycogen was stained within the liver with PAS stainings as described previously . Shortly, tissue sections were incubated in 0.8% periodic acid (Sigma, Deisenhofen, Germany) followed by an incubation in Schiff's reagent (Sigma, Deisenhofen, Germany). Sections were counterstained with Shandon Gill3 Hematoxylin (Thermo Scientific, Cheshire, UK).

2.13 5-bromo-2-deoxyuridine (BrdU) staining.

Two hours prior to sacrifice, mice were injected with 0.1 µg/g body weight (bw) BrdU. Positive cells were visualized immunohistochemically. Staining for BrdU was carried out using

a mouse BrdU mAb (Vector Laboratories, CA) diluted in sample diluent (Dako, Glostrup, Denmark). After incubation with labeled polymer-HRP-anti-mouse mAb (Dako envision, Glostrup, Denmark), the signal was developed with AEC Substrate (Dako, Glostrup, Denmark). Samples were counterstained with Shandon Gill3 Hematoxylin (Thermo Scientific, Cheshire, UK).

2.14 Preparation of microsomes.

Mice livers were homogenized in 10 mM KH_2PO_4 , pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, 0.1% (w/v) BSA and 1 mM DTT at 4°C. The homogenate was centrifuged at 750 x g for 15 min and the supernatant was again centrifuged until the pellet remained pale (normally 3-5 centrifugation steps). The pellet was discarded. Subsequently the supernatant was centrifuged at 10,300 x g for 20 min. The pellet was discarded and the supernatant was centrifuged at 137,000 x g for 70 min. The supernatant was discarded and the pellet was resuspended in buffer and centrifuged again at 137,000 x g for 70 min. The final pellet was resuspended in buffer and stored at -80°C. Samples were taken for measuring protein concentration using the BCA Kit (Pierce, Rockford, IL). Equal amounts of protein were analyzed with Western blot analysis using a polyclonal CYP2E1 antibody (Abcam, Cambridge, UK) as described above.

2.15 Thiobarbituric acid reactive substances (TBARS) quantifications.

TBARS were used as an index of the extent of lipid peroxidation. 1 mg liver tissue homogenate (100 μl) was mixed with 200 μl ice cold 10% (w/v) trichloroacetic acid to precipitate proteins. After incubation (15 min on ice) and centrifugation (2,200 x g, 15 min, 4°C) 200 μl supernatant was mixed with equal volume 0.67% (w/v) thiobarbituric acid (TBA) and incubated for 10 min at 100°C. After cooling, the lipid peroxidation product

malondialdehyde (MDA) was measured at 532 nm using 1,1,3,3-tetramethoxypropane as standard.

3. RESULTS

3.1 Upregulation of sIL-6R upon CCl₄ induced liver damage

Acute liver damage was induced with a single injection of 3 ml/kg body weight of a 20% (v/v) CCl₄ solution. Serum levels of sIL-6R as measured by ELISA were significantly elevated after CCl₄ treatment. As shown in Fig. 1, a more than two-fold increase in endogenous sIL-6R levels was observed directly after damage induction. Elevated sIL-6R levels were maintained throughout 48 h, indicating that IL-6 trans-signaling was induced upon liver damage by CCl₄ treatment.

3.2 Quantification of CCl₄ induced liver damage

Liver damage after CCl₄ treatment was quantified by measuring serum ALT and AST levels (Fig. 2A, B). To specifically block IL-6 trans-signaling, mice were injected with 250 µg sgp130Fc i.p. per mouse 18 h before CCl₄ treatment. Serum levels of sgp130Fc levels ranged between 35-55 µg/ml as verified by ELISA measurements and therefore were shown to be high enough to inhibit the activity of the IL-6/sIL-6R complexes throughout the course of the experiment (Suppl. Fig 1). Treatment of the mice with the designer cytokine Hyper-IL-6 led to no significant changes in serum ALT levels as compared to CCl₄ treatment alone. It should be noted, however, that Hyper-IL-6 was only injected once 18 h prior to CCl₄ treatment. Since the half-life of Hyper-IL-6 is in the range of 24 h, little of the cytokine would be expected to be left in the circulation after 48 h. We therefore believe that in order to rigorously test a beneficial effect of Hyper-IL-6 during liver damage, experiments with daily injections of Hyper-IL-6 protein or with genetic delivery via an adenovirus should be performed.

CCl₄ treatment increased ALT and AST levels after 24 and 48 h. When IL-6 trans-signaling was blocked by injection of recombinant sgp130Fc protein, ALT and AST levels increased dramatically after 24 h and elevated levels were maintained at 48 h. Injection of Hyper-IL-6 resulted in a slight decrease of liver damage induced by CCl₄ treatment after 48 h. Histological examination of the overall liver damage 48 h after CCl₄ administration showed smaller necrotic areas in CCl₄ treated mice compared to sgp130Fc pretreated mice (Fig. 2C, a, b, c, d). Quantification of liver damage 48 h after CCl₄-induced liver damage indicated that only 33.2% of the liver was necrotic in CCl₄ treated mice compared to 58.6% in sgp130Fc pretreated mice (Fig 2E). No significant difference was seen in the number of apoptotic nuclei as measured by TUNEL staining (Fig. 2C, e, f). Using DAPI staining, we noted less intact nuclei and extended necrotic areas in sgp130Fc pretreated animals (Fig. 2C, g, h). The reduction of DAPI positive hepatocytes is shown in Fig. 2D. Interestingly, after 6 and 24 h, IL-6 levels were significantly higher in sgp130Fc pretreated mice as compared to control animals (Suppl. Fig. 2). These results indicated that endogenous IL-6 trans-signaling was protective after CCl₄ treatment.

3.3 Quantification of oxidative damage

Thiobarbituric acidic reactive substances (TBARS) are indicators of lipid peroxidation. TBARS were elevated in liver tissues of sgp130Fc treated animals 4 and 6 h after CCl₄ treatment (Fig. 3A). This enhancement of lipid peroxidation could be reversed when mice were treated with Hyper-IL-6 prior to CCl₄ (data not shown). Moreover, after CCl₄ treatment, uric acid and potassium levels in the blood were elevated upon sgp130Fc treatment and lowered upon Hyper-IL-6 treatment (Suppl. Fig. 3). Since uric acid and potassium are markers for oxidative and general cell damage these data confirm that oxidative cell damage is higher when IL-6 trans-signaling is blocked.

3.4 Expression levels of Cyp2E1 in the livers of CCl₄ treated mice

Liver damage by CCl₄ depends on biotransformation catalyzed by cytochrome P450 2E1 (Cyp2E1). To measure protein expression levels of Cyp2E1, microsomes were prepared and Western blots were carried out. Fig. 3B shows that protein expression of Cyp2E1 was strongly induced 24 and 48 h after CCl₄ treatment as compared to 6 h after CCl₄ treatment. This is interesting since in a recent publication, Horiguchi and coauthors showed that upon CCl₄ treatment of mice, CYP2E1 expression was markedly downregulated at 6 and 12 h post CCl₄ treatment. Notably, treatment of the animals with sgp130Fc did not affect protein levels of Cyp2E1. Therefore, higher liver damage in sgp130Fc treated mice did not result from different Cyp2E1 levels in the liver.

3.5 gp130 signaling in sgp130Fc treated animals

Treatment of mice with CCl₄ led to activation of the STAT3 pathway after 2 h with a peak after 4 h as measured by phosphorylation of STAT3 (Fig. 4A). Treatment of the mice with the sgp130Fc protein resulted in a strong reduction in phosphorylated STAT3 protein in CCl₄-treated mice. Interestingly, only partial blockade of STAT3 activation was achieved by sgp130Fc treatment indicating that gp130 on liver cells was also stimulated via the membrane bound IL-6R upon after CCl₄ treatment. It should be noted that treatment of mice with sgp130Fc alone did not affect phosphorylation of the STAT3 protein. Liver cell proliferation was quantified by BrdU staining. 2 h prior to sacrifice, mice were injected with BrdU and liver sections were analyzed immunohistochemically. Quantification of positive cells revealed that liver cell proliferation depended at least in part on signaling via IL-6/sIL-6R, since a blockade of this pathway significantly reduced the number of proliferating cells (Fig. 4B).

3.6 Role of neutrophils in CCl₄ damaged livers

It has been documented that the presence of neutrophils has an impact on liver damage in different models including CCl₄ induced liver damage . To determine the role of neutrophils after CCl₄ damage, we depleted neutrophils in control and sgp130Fc pretreated mice prior to CCl₄ injection and investigated the impact of neutrophil depletion on the severity of liver damage. Mice were treated with anti Ly-6G mAb 18 h before CCl₄ injection and sacrificed 24 h after the insult. Depletion of neutrophils resulted in reduced ALT levels in CCl₄ injected mice irrespective of sgp130Fc pre-treatment (Fig. 5). Although sgp130Fc pretreatment led to higher ALT levels in the absence or presence of neutrophils, the difference in ALT levels between neutrophil depleted and non-depleted animals was similar in sgp130Fc untreated and treated mice, indicating that part of the liver damage observed in CCl₄ treated animals was caused by neutrophils although they do not explain the observed higher damage in the sgp130Fc treatment group.

3.7 Impact of IL-6 trans-signaling on glycogen content within the liver

CCl₄ exposure leads to activation of glycogen phosphorylase, and causes glycogen depletion in the liver . As shown in Fig. 6, no glycogen was detectable in liver sections 24 h after liver damage. 48 h after treatment, glycogen was restored in the livers of CCl₄ treated animals, whereas sgp130Fc pretreated mice showed very little glycogen in the liver. These findings indicate that IL-6 trans-signaling has a significant impact on glycogen metabolism after CCl₄ induced liver damage.

4. DISCUSSION

In the present study we show that the response of the liver to an acute CCl₄ insult is strongly influenced by IL-6 trans-signaling via the sIL-6R. Early after CCl₄ treatment, sIL-6R levels were significantly increased. The protein sgp130 is a natural, specific inhibitor of IL-6 trans-signaling and we have developed a sgp130Fc fusion protein, which blocks IL-6 trans-signaling without affecting classical IL-6 signaling via the membrane bound IL-6R. In this study we show that blockade of the IL-6 trans-signaling pathway with sgp130Fc leads to an increase in state levels of IL-6 at early time points after the injury. This increase in IL-6 might be explained by the increased half-life of IL-6 prolonged by the formation of sgp130F/sIL-6R/IL-6 immune complexes. A similar effect has been found after the administration of IL-6R neutralizing antibodies to patients with rheumatoid arthritis and Castleman disease.

In gene deficient mice it has been shown that CCl₄ mediated damage was more severe in the absence of IL-6. We now demonstrate that liver damage is increased when only IL-6 trans-signaling is blocked. Different parameters of liver damage were quantified. Serum ALT and AST levels were found to be significantly higher in the presence of the sgp130Fc protein. Increased liver damage was also visible morphologically in HE stainings showing massive necrotic areas. DAPI stainings showed less intact nuclei than in livers of control mice, indicating necrotic tissue damage.

An additional aspect of CCl₄ induced liver injury is the induction of inflammation and oxidative damage. Oxidative stress is commonly quantified via the amount of lipid peroxidation in liver tissue. In the present study we show increased lipid peroxidation when IL-6 trans-signaling was blocked. Similar results were obtained in IL-6 deficient mice. Other parameters for oxidative stress or common cell damage are uric acid and potassium, which

play a critical role in antioxidant defense and lipid peroxidation in human blood plasma . These factors were upregulated when IL-6 trans-signaling was blocked indicating that this signaling pathway is important in the defense of the body to oxidative stress.

Investigating possible explanations for these observations we focused on the impact of infiltrated neutrophils within the liver. Recently it was found that in a kidney damage model, the number of infiltrating neutrophils and the tissue damage was lower in IL-6 deficient mice . For CCl₄ induced liver damage it was found that neutrophils participate in CCl₄ induced liver damage, which was reduced upon depletion of neutrophils . We have demonstrated before that less infiltrating neutrophils were detected in acute inflammation when the IL-6 trans-signaling pathway was blocked . In CCl₄ induced liver damage we confirmed that depletion of neutrophils before CCl₄ treatment reduced the liver damage. However, in sgp130Fc pretreated mice, ALT levels and other markers of liver damage were higher than in CCl₄ treated animals, whereas neutrophil levels remained unchanged. We therefore hypothesize that the effect of IL-6 trans-signaling on the extent of the induced liver damage is not dependent on neutrophils.

It should be noted that pretreatment with sgp130Fc very likely may have reduced STAT3 activation not only in the liver but may also in immune cells such as macrophages and neutrophils. Inhibition of STAT3 in macrophages and neutrophils may therefore account for the increased IL-6 levels because in a recent study, myeloid specific STAT3 conditional knockout had resulted in elevated IL-6 levels after CCl₄ treatment as compared to wildtype mice .

24 h after CCl₄ treatment glycogen within the liver was consumed but was replenished 48 h after CCl₄ treatment. However, when IL-6 trans-signaling was blocked, glycogen

replenishment was significantly delayed. Interestingly, we have recently noted that glycogen consumption was blocked 24 h after D-galactosamine damage in sgp130Fc transgenic mice underlining the different mechanism of liver damage by CCl₄.

In earlier reports we have demonstrated the potential of the IL-6/sIL-6R complex to induce liver regeneration. In the present study, we show that the concentration of sIL-6R increased after CCl₄ administration. Furthermore, by blocking endogenous IL-6 trans-signaling we demonstrate that this pathway is important for the response of the liver to chemical damage. Importantly, the role of STAT3 has most recently been addressed in two elegant studies using conditional gene ablation. The authors showed that hepatocyte specific STAT3 knockout in mice resulted in enhanced liver injury upon treatment of the mice with CCl₄ but decreased liver regeneration in response to partial hepatectomy ablation. These studies clearly demonstrated that the role of STAT3 in the response of the liver to injury and damage is complex. Moreover, it should be noted that other gp130-induced signaling pathways such as the AKT pathway may also be important for the integrity of the liver upon injury and damage. Since IL-6 neutralizing strategies have been approved for the clinic in Europe and the US for the treatment of inflammatory diseases, it will be important to monitor, which activities of IL-6 – when blocked – might prove harmful to the organism.

ACKNOWLEDGEMENTS

The authors thank Nadja Timm, Nico Schneider, Elsbeth Schulz and Stefanie Schnell for excellent technical assistance. This work was funded by grants from the Deutsche Forschungsgemeinschaft, Bonn, Germany (SFB841, project C1) and by the Cluster of Excellence 'Inflammation at Interfaces'.

- Interleukin-6 (IL-6) plays an important role in liver regeneration and protection against liver damage. In addition to IL-6 classic signaling via membrane bound receptor (mIL-6R), IL-6 signaling can also be mediated by soluble IL-6R (sIL-6R) thereby activating cells that do not express membrane bound IL-6R. This process has been named trans-signaling.
- A soluble gp130 protein (sgp130Fc) exclusively inhibits IL-6 trans-signaling whereas an IL-6/sIL-6R fusion protein (Hyper-IL-6) mimics IL-6 trans-signaling.
- Using these tools we investigate the role of IL-6 trans-signaling in liver damage. Blockade of IL-6 trans-signaling during liver damage led to higher liver damage.
- Depletion of neutrophils resulted in reduced liver damage.
- IL-6 trans-signaling was important for refilling of hepatocyte glycogen stores, which were depleted 24 h after CCl₄ treatment.
- We conclude that IL-6 trans-signaling via the soluble IL-6R is important for the physiologic response of the liver to CCl₄ induced chemical damage.

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FIGURE LEGENDS

Figure 1. Serum levels of soluble IL-6Receptor (sIL-6R) after CCl₄ induced liver damage. C57Bl/6N mice were injected i.p. with a single dose of 3 ml/kg BW of a 20 % (v/v) CCl₄ solution (black bars) or mock treated (white bars). Serum levels of sIL-6R were measured by ELISA at the indicated time points. sIL-6R levels were significantly increased direct ($P < 0.0001$), 24 hours ($P < 0.005$) and 48 hours ($P < 0.0001$) after CCl₄ treatment.

Figure 2. CCl₄ induced liver damage increases when IL-6 trans-signaling is blocked. C57Bl/6N mice were injected i.p. with a single dose of sgp130Fc to block IL-6 trans-signaling before treated with CCl₄. (A) ALT and (B) AST were measured in blood serum 24 and 48 hours after CCl₄ treatment. ALT ($P < 0.0001$) and AST ($P < 0.05$) levels were significantly higher when IL-6 trans-signaling was blocked with sgp130Fc. (C) Histological evaluations of liver sections 48 hours after CCl₄ injection. (a) Hematoxylin Eosin stainings (10 fold magnification) of CCl₄ only and (b) of sgp130Fc pretreated mice. The damaged area is circled for quantification with the ImageJ-Software. (c) Hematoxylin Eosin stainings (20 fold magnification) of CCl₄ only and (d) of sgp130Fc pretreated mice, (e) Tunel stainings (20 fold magnification) of CCl₄ only and (f) of sgp130Fc pretreated mice, (g) DAPI stainings (40 fold magnification) of CCl₄ only and (h) of sgp130Fc pretreated mice. Damage was more severe in sgp130 pretreated animals, but the occurrence of apoptotic events is comparable. (D) Quantification of HE stainings show more necrotic area (circled in a, b) in sgp130Fc pretreated mice ($P < 0.005$) when compared to CCl₄ treated mice. (E) Quantification of Tunel stainings show comparable apoptotic events in sgp130Fc pretreated and CCl₄ treated mice. (F) Evaluation of DAPI stainings show that more nuclei are intact in CCl₄ treated animals in comparison to sgp130Fc pretreated animals ($P < 0.0001$).

Figure 3. Quantification of oxidative damage. (A) Evaluation of oxidative stress by TBARS quantification. TBARS were measured in total protein lysates 0, 2, 4 and 6 hours after intoxication with CCl₄. Values were significantly increased in sgp130Fc pretreated mice (grey bars) 4 and 6 hours post CCl₄ injection when compared to CCl₄ only treated mice ($P < 0.05$). (B) Western blot for Cyp2E1 of microsomal preparations 48 hours post CCl₄ injection.

Figure 4. STAT3 phosphorylation and liver cell proliferation is impaired in mice with blocked IL-6 trans-signaling. (A) Western blots showing that STAT3 phosphorylation was impaired when IL-6 transsignaling was blocked by sgp130Fc as compared to mice treated with CCl₄ only. (B) Quantification of western blots show reduced STAT3 phosphorylation 4 hours after CCl₄ injection in sgp130Fc pretreated mice. (C) Quantification of BrdU stainings of liver section 48 hours after CCl₄ injection showing less BrdU-positive nuclei in sgp130Fc pretreated mice (grey bars) when compared to mice treated with CCl₄ only (black bars) ($P < 0.0001$).

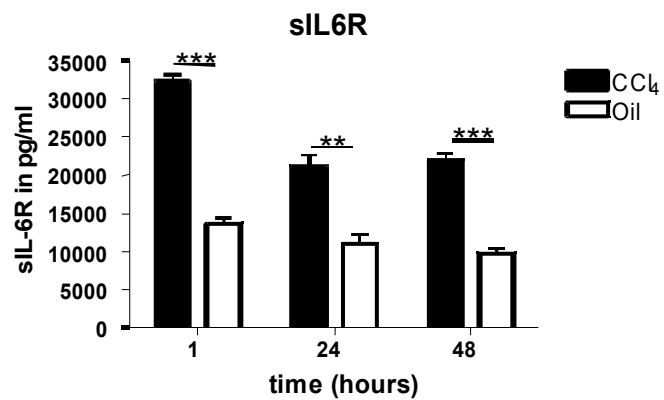
Figure 5. Blockade of IL-6 trans-signaling is independent of neutrophils. (A) Neutrophils were depleted with the anti-Ly6G/Ly6C monoclonal antibody before CCl₄ injection. Liver damage was quantified via serum ALT levels 24 hours post CCl₄ treatment. ALT values were significantly reduced in the sera of neutrophil depleted animals ($P < 0.005$). (B) Quantitative blood flow cytometric analysis of neutrophils in percent at the indicated time points after CCl₄ induced liver damage was carried out in C57Bl/6N mice treated with CCl₄ after previous injection of sgp130Fc or mock treatment. (C) Quantitative blood flow cytometric analysis of neutrophils in percent at the indicated time points after CCl₄ induced liver damage was performed in C57Bl/6N and sgp130Fc transgenic mice treated with CCl₄.

Fig. 6. Glycogen staining in livers upon CCl₄ damage. PAS staining of liver sections 24 and 48 hours after CCl₄ induced liver damage showed decelerated glycogen restoration in sgp130Fc pretreated animals 48 hours after CCl₄ injection when compared to mice treated with CCl₄ only. (a) CCl₄ only 24 hours and (b) CCl₄ only 48 hours post CCl₄. (c) sgp130Fc pretreated 24 hours and (d) sgp130Fc pretreated 48 hours post CCl₄. (e) Mock (oil only) treated for 24 hours and (f) 48 hours.

Supplementary Figure 1. C57Bl/6 mice were injected with 250 µg sgp130Fc protein i.p. and serum sgp130Fc levels were measured via ELISA.

Supplementary Figure 2. IL-6 was measured in blood serum via ELISA at the indicated time points. IL-6 levels increased 24 hours after CCl₄ treatment. The rise was significantly higher in sgp130Fc pretreated mice (grey bars) from the time 6 hours after CCl₄ treatment up to 24 hours after treatment when compared to mice treated with CCl₄ only (P < 0.0001).

Supplementary Figure 3. (A) Uric acid was measured in blood serum 48 hours after CCl₄ injection. Levels increased significantly when IL-6 trans-signaling was blocked via sgp130Fc (grey bars) as compared to mice treated with CCl₄ only (black bars) (P < 0.05) and Hyper-IL-6 pretreated mice (striped grey bars) (P < 0.005). (B) Potassium levels were measured in blood sera 48 hours after CCl₄ induced liver damage. sgp130Fc pretreated mice (grey bars) exhibited slightly higher potassium levels, whereas the other modulations of the IL-6 signal pathway did not have any effects on the potassium level.



ACCEPTED MANUSCRIPT

Fig. 1

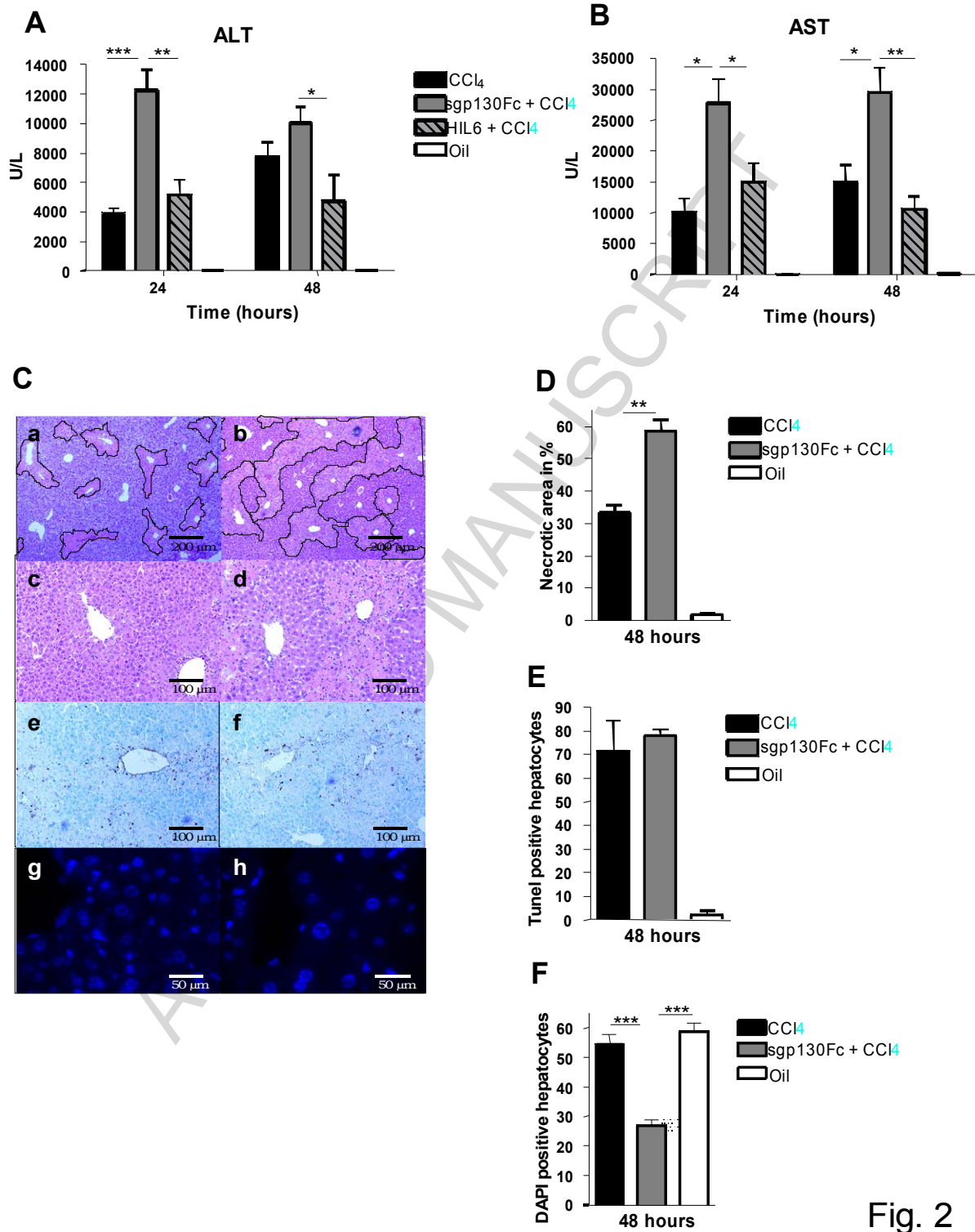


Fig. 2

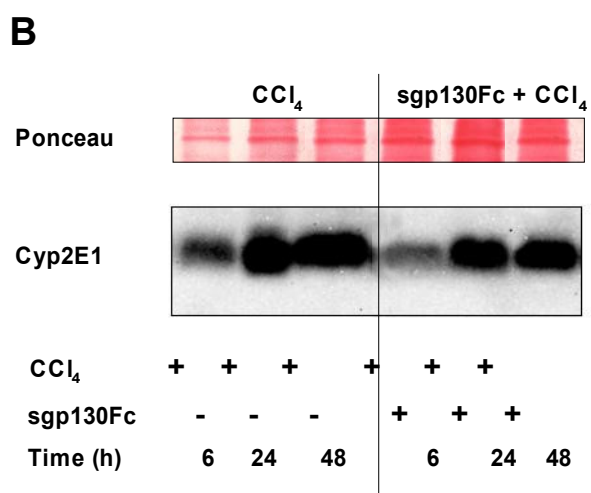
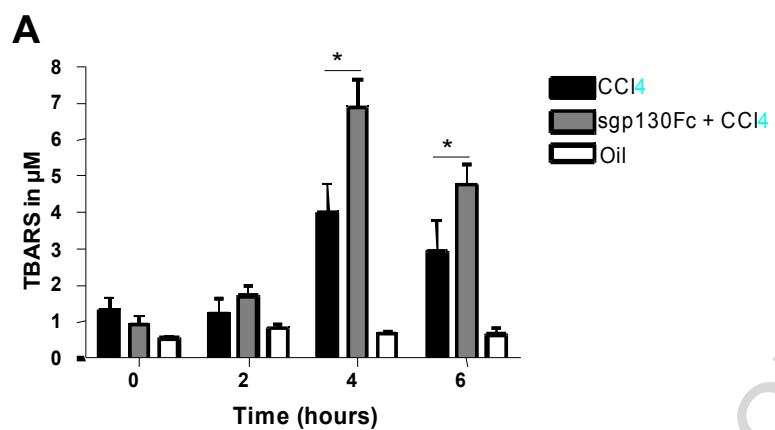


Fig. 3

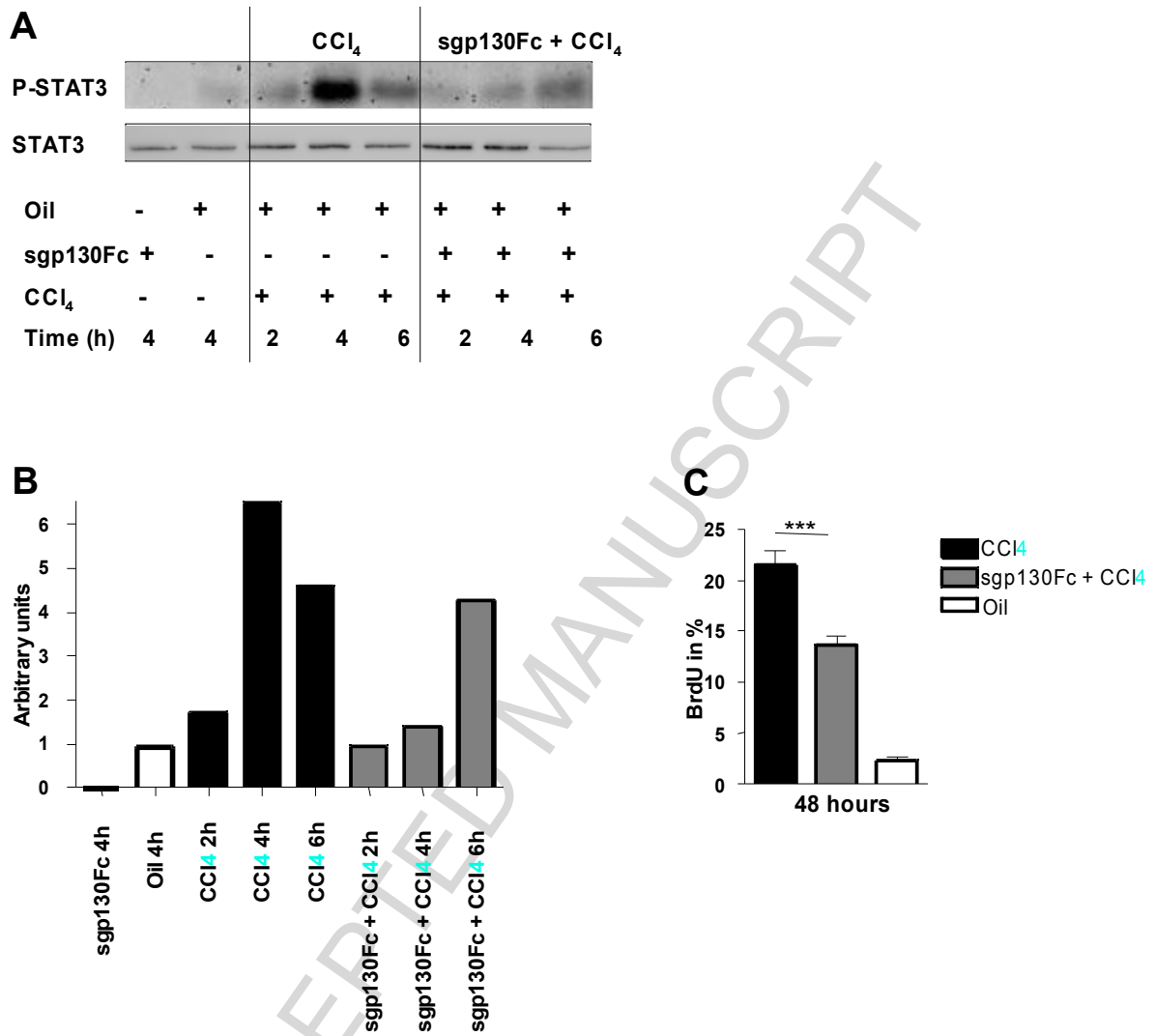


Fig. 4

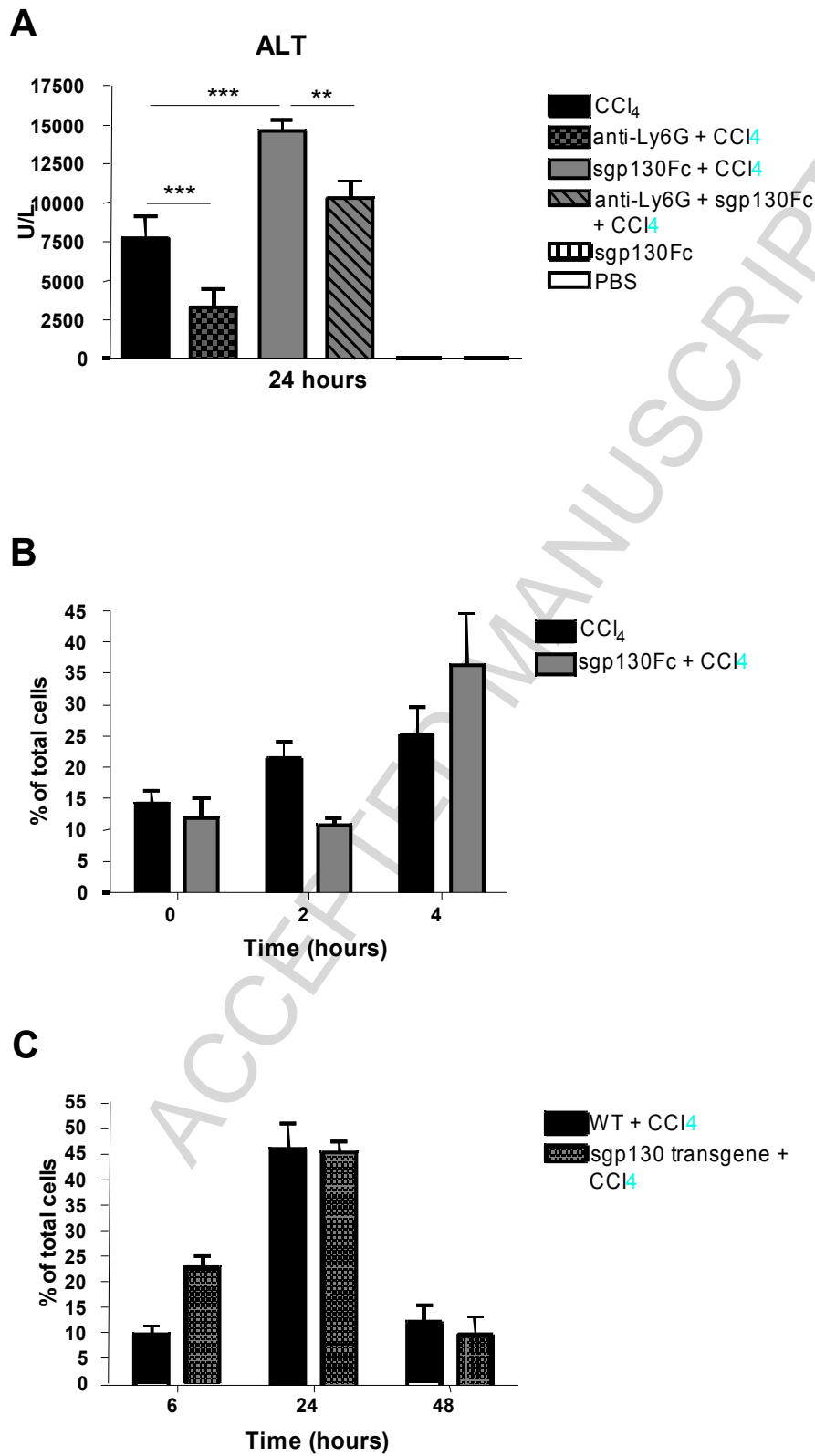


Fig. 5

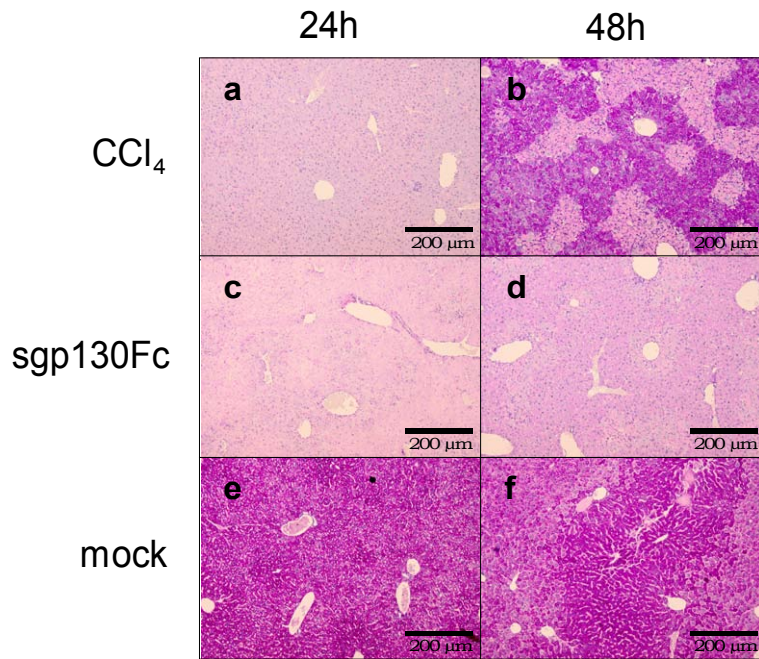
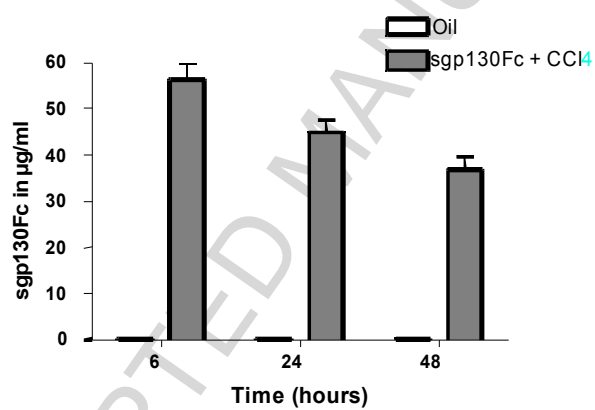
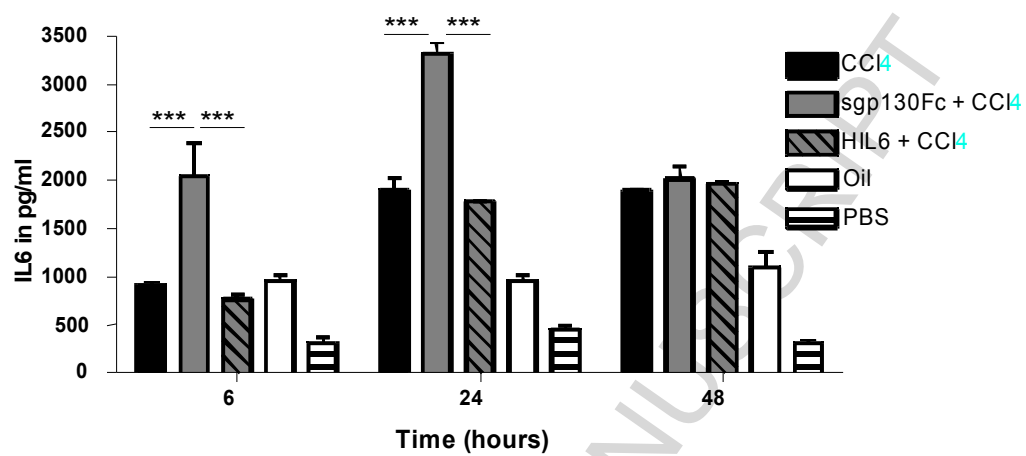


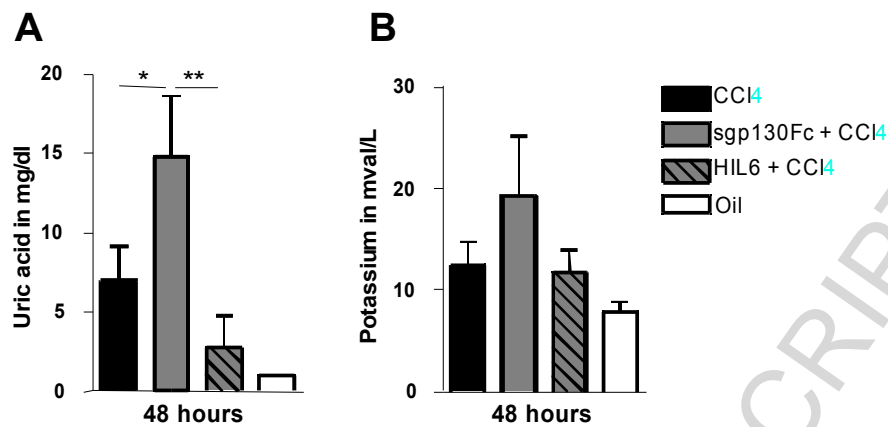
Fig. 6



Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3