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Role of IL-6 trans-signaling in CCl_4 induced liver damage

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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 CCl_4 induced liver damage. Blockade of IL-6 trans-signaling during CCl_4 induced liver damage led to higher liver damage, although induction of Cyp4502E1 and thus bioactivation of CCl_4 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 CCl_4 treatment. We conclude that IL-6 trans-signaling via the soluble IL-6R is important for the physiologic response of the liver to CCl_4 induced chemical damage.

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 [1,2].

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 [3,4]. We developed Hyper-IL-6, a fusion protein of IL-6 bound to the sIL-6R which mimics IL-6 trans-signaling [5]. *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 [6–8].

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 [9]. This sgp130Fc protein was used to demonstrate that endogenous IL-6 trans-signaling was important for chronic inflammation states [10,11] and colon cancer [12]. Recently, we could demonstrate that sgp130Fc reduced glycogen consumption in the liver of animals treated with D-galactosamine [13].

CCl_4 is a hepatotoxin that causes direct hepatocyte damage by altering the permeability of cellular, lysosomal and mitochondrial membranes [14]. 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 [15–17]. Recently, it has been shown that CCl_4 not only causes primary liver necrosis, but also hepatocyte apoptosis [18].

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 [19].

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.

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2. Materials and methods

2.1. Animals and CCl_4 treatment

Male C57Bl/6 N mice were kept at a 12-h light-dark cycle under standard conditions and provided with food and water *ad libitum*. Sgp130 transgenic animals were generated [20] and treated identically to C57Bl/6 N 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 CCl_4 (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 CCl_4 treatment. sgp130Fc levels were measured via Enzyme-linked immunoabsorbent assays (ELISA) in the serum of the mice as described [20] using a human gp130 Elisa Kit (DuoSet human gp130 ELISA Kit, R&D Systems, Wiesbaden, Germany) according to the manufacturer's 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 [5] i.p. 18 h prior to CCl_4 treatment.

2.4. Neutrophil depletion

As described previously [20], 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 CCl_4. 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 1500 rpm for 5 min at 4 °C, supernatant was discarded and pellet was immediately resuspended in 100 μl 1 × 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 [13]. 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 X-ray-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 [13]. 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*g* for 15 min and the supernatant was again centrifuged until the pellet remained pale (normally 3–5 centifugation steps). The pellet was discarded. Subsequently the supernatant was centrifuged at 10,300*g* for 20 min. The pellet was discarded and the supernatant was centrifuged at 137,000*g* for 70 min. The supernatant was discarded and the pellet was resuspended in buffer and centrifuged again at 137,000*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 [21]. 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 (2200*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 CCl_4 induced liver damage

Acute liver damage was induced with a single injection of 3 ml/kg body weight of a 20% (v/v) CCl_4 solution. Serum levels of sIL-6R as measured by ELISA were significantly elevated after CCl_4 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 CCl_4 treatment.

3.2. Quantification of CCl_4 induced liver damage

Liver damage after CCl_4 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 CCl_4 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 [20,22] throughout the course of the experiment (Suppl. Fig. 1). Treatment of the mice with the designer

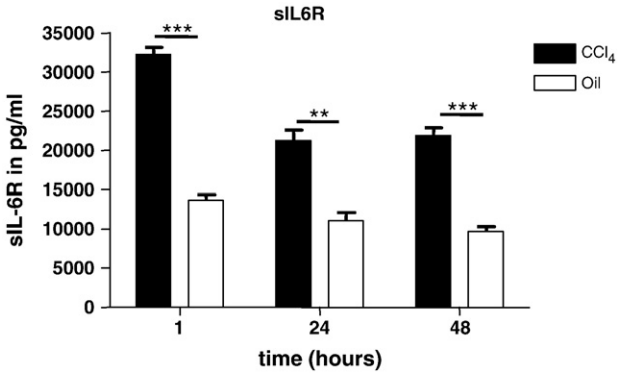

Fig. 1. Serum levels of soluble IL-6Receptor (sIL-6R) after CCl_4 induced liver damage. C57Bl/6 N mice were injected i.p. with a single dose of 3 ml/kg BW of a 20% (v/v) CCl_4 solution (black bars) or mock treated (white bars). Serum levels of sIL-6R were measured by ELISA at the indicated time points. sIL-6R levels were significantly increased direct ($P<0.0001$), 24 h ($P<0.005$) and 48 h ($P<0.0001$) after CCl_4 treatment.

cytokine Hyper-IL-6 led to no significant changes in serum ALT levels as compared to CCl_4 treatment alone. It should be noted, however, that Hyper-IL-6 was only injected once 18 h prior to CCl_4 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 [8] should be performed.

CCl_4 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 CCl_4 treatment after 48 h. Histological examination of the overall liver damage 48 h after CCl_4 administration showed smaller necrotic areas in CCl_4 treated mice compared to sgp130Fc pretreated mice (Fig. 2C, a, b, c, d). Quantification of liver damage 48 h after CCl_4-induced liver damage indicated that only 33.2% of the liver was necrotic in CCl_4 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 CCl_4 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 CCl_4 treatment (Fig. 3A). This enhancement of lipid peroxidation could be reversed when mice were treated with Hyper-IL-6 prior to CCl_4 (data not shown). Moreover, after CCl_4 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 [23] 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 CCl_4 treated mice

Liver damage by CCl_4 depends on biotransformation catalyzed by cytochrome P450 2E1 (Cyp2E1). To measure protein expression levels

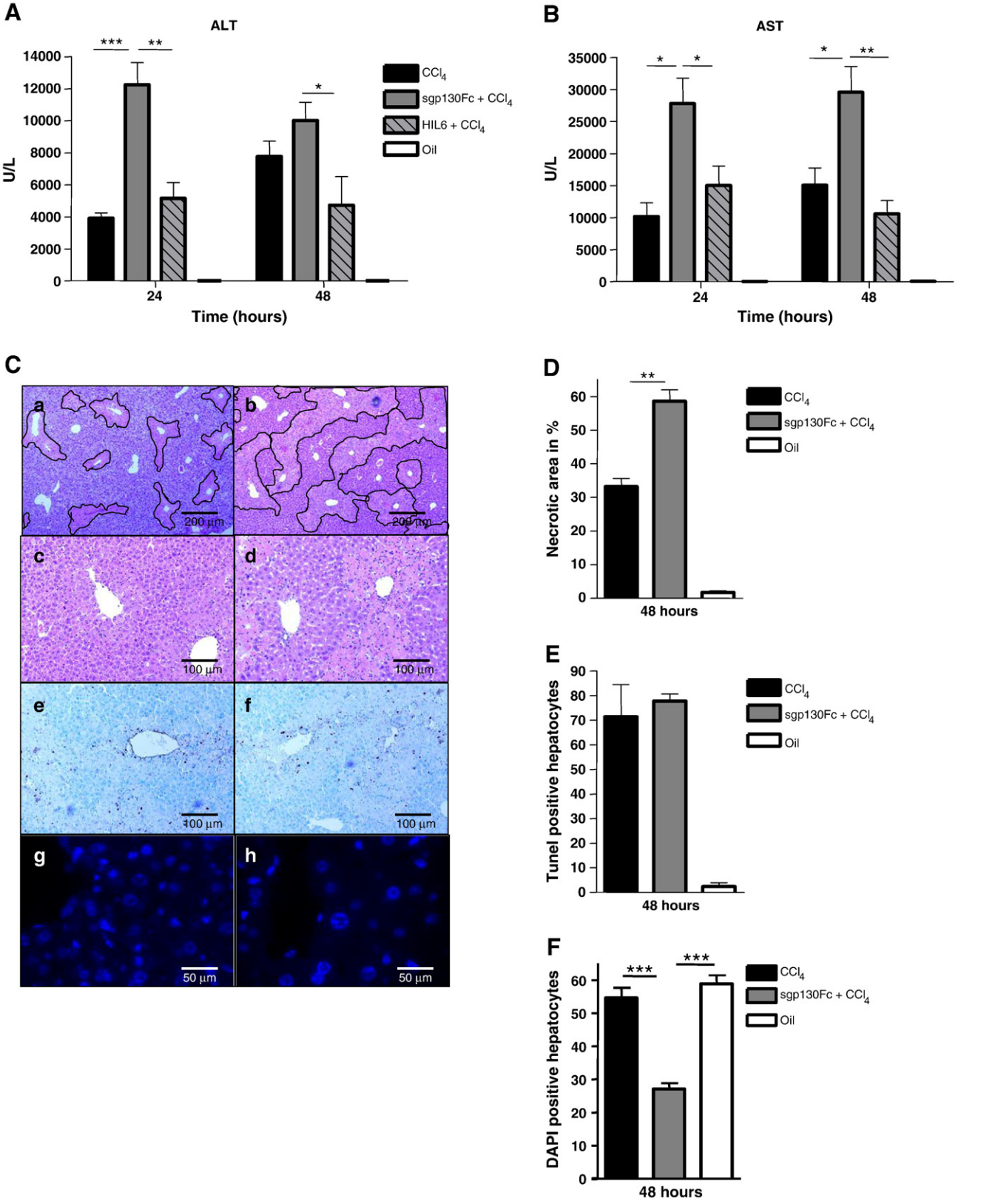

Fig. 2. CCl_4 induced liver damage increases when IL-6 trans-signaling is blocked. C57Bl/6 N mice were injected i.p. with a single dose of sgp130Fc to block IL-6 trans-signaling before treated with CCl_4. (A) ALT and (B) AST were measured in blood serum 24 and 48 h after CCl_4 treatment. ALT ($P<0.0001$) 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 h after CCl_4 injection. (a) Hematoxylin–eosin stainings (10-fold magnification) of CCl_4 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 CCl_4 only and (d) of sgp130Fc pretreated mice, (e) Tunel stainings (20 fold magnification) of CCl_4 only and (f) of sgp130Fc pretreated mice, (g) DAPI stainings (40 fold magnification) of CCl_4 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 CCl_4 treated mice. (F) Evaluation of DAPI stainings show that more nuclei are intact in CCl4 treated animals in comparison to sgp130Fc pretreated animals ($P< 0.0001$).

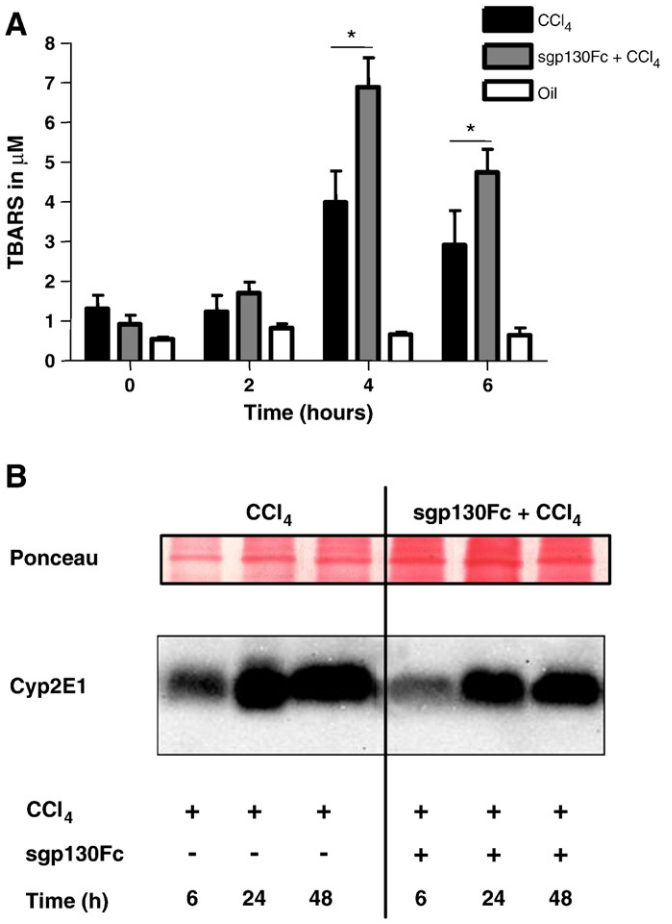

Fig. 3. Quantification of oxidative damage. (A) Evaluation of oxidative stress by TBARS quantification. TBARS were measured in total protein lysates 0, 2, 4 and 6 h after intoxication with CCl_4. Values were significantly increased in sgp130Fc pretreated mice (grey bars) 4 and 6 h post CCl_4 injection when compared to CCl_4 only treated mice ($P<0.05$). (B) Western blot for Cyp2E1 of microsomal preparations 48 h post CCl_4 injection.

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 CCl_4 treatment as compared to 6 h after CCl_4 treatment. This is interesting since in a recent publication, Horiguchi and coauthors showed that upon CCl_4 treatment of mice, CYP2E1 expression was markedly downregulated at 6 and 12 h post CCl_4 treatment [24]. 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 CCl_4 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 CCl_4-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 CCl_4 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 CCl_4 damaged livers

It has been documented that the presence of neutrophils has an impact on liver damage in different models including CCl_4 induced liver damage [25]. To determine the role of neutrophils after CCl_4 damage, we depleted neutrophils in control and sgp130Fc pretreated mice prior to CCl_4 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 CCl_4 injection and sacrificed 24 h after the insult. Depletion of neutrophils resulted in reduced ALT levels in CCl_4 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 CCl_4 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

CCl_4 exposure leads to activation of glycogen phosphorylase, and causes glycogen depletion in the liver [26,27]. 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 CCl_4 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 CCl_4 induced liver damage.

4. Discussion

In the present study we show that the response of the liver to an acute CCl_4 insult is strongly influenced by IL-6 trans-signaling via the sIL-6R. Early after CCl_4 treatment, sIL-6R levels were significantly increased. The protein sgp130 is a natural, specific inhibitor of IL-6 trans-signaling [9] 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 [28]. 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 [29].

In gene deficient mice it has been shown that CCl_4 mediated damage was more severe in the absence of IL-6 [19,30]. 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 CCl_4 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 [19]. 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

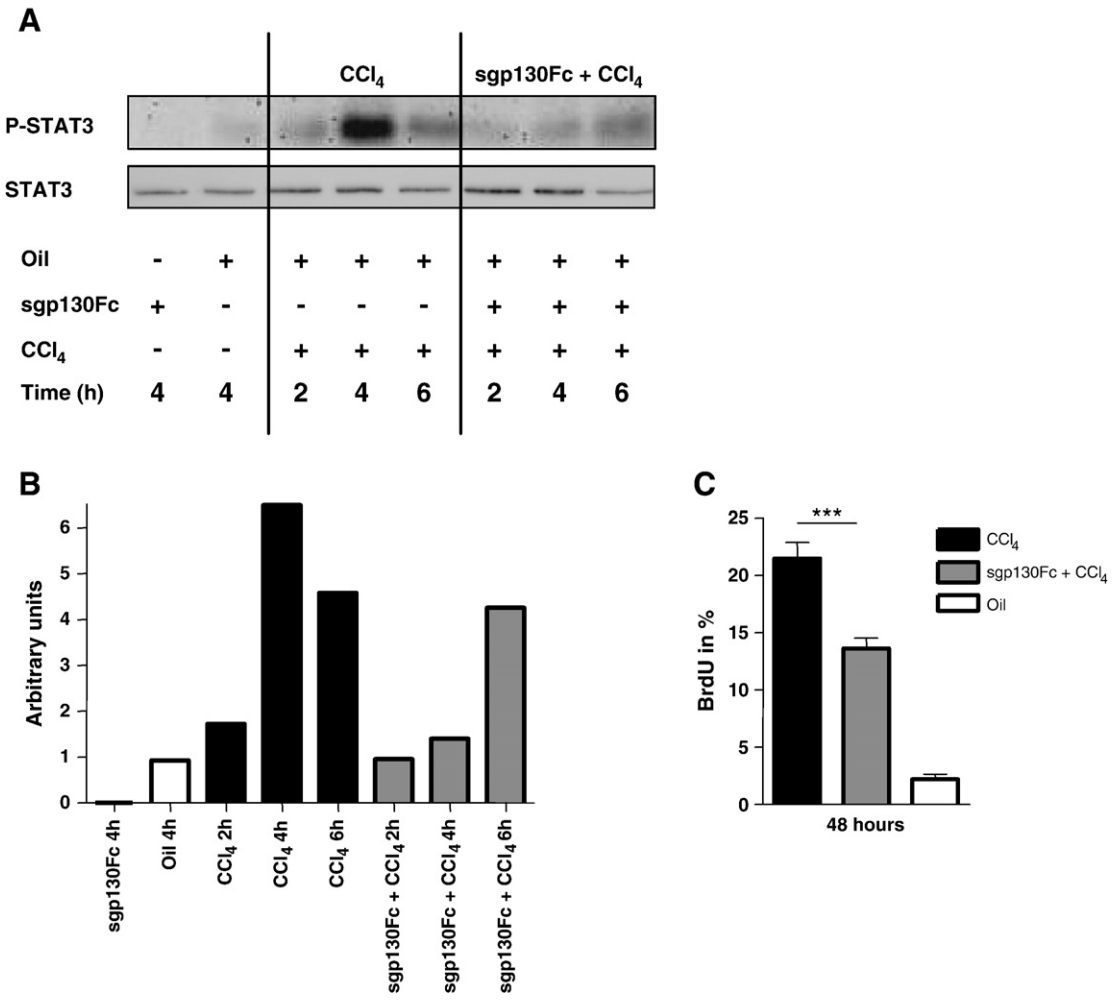

Fig. 4. STAT3 phosphorylation and liver cell proliferation is impaired in mice with blocked IL-6 trans-signaling. (A) Western blots showing that STAT3 phosphorylation was impaired when IL-6 transsignaling was blocked by sgp130Fc as compared to mice treated with CCl4 only. (B) Quantification of western blots show reduced STAT3 phosphorylation 4 h after CCl_4 injection in sgp130Fc pretreated mice. (C) Quantification of BrdU stainings of liver section 48 h after CCl4 injection showing less BrdU-positive nuclei in sgp130Fc pretreated mice (grey bars) when compared to mice treated with CCl4 only (black bars) ($P<0.0001$).

[31]. 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 [32]. For CCl_4 induced liver damage it was found that neutrophils participate in CCl_4 induced liver damage, which was reduced upon depletion of neutrophils [25]. We have demonstrated before that less infiltrating neutrophils were detected in acute inflammation when the IL-6 trans-signaling pathway was blocked [20,22]. In CCl_4 induced liver damage we confirmed that depletion of neutrophils before CCl_4 treatment reduced the liver damage. However, in sgp130Fc pretreated mice, ALT levels and other markers of liver damage were higher than in CCl_4 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 CCl_4 treatment as compared to wildtype mice [24].

24 h after CCl_4 treatment glycogen within the liver was consumed but was replenished 48 h after CCl_4 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 CCl_4 [13].

In earlier reports we have demonstrated the potential of the IL-6/sIL-6R complex to induce liver regeneration [6–8]. In the present study, we show that the concentration of sIL-6R increased after CCl_4 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 [24,33]. The authors showed that hepatocyte specific STAT3 knockout in mice resulted in enhanced liver injury upon treatment of the mice with CCl_4 but decreased liver regeneration in response to partial hepatectomy ablation [24,33]. 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

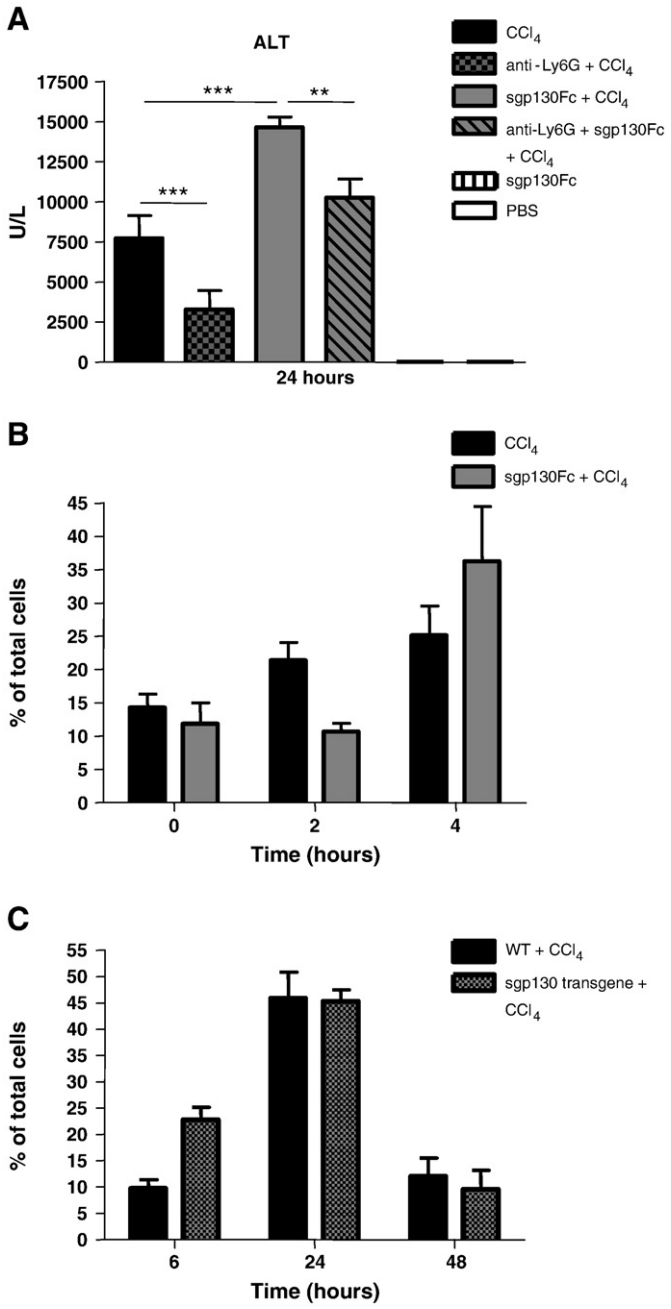

Fig. 5. Blockade of IL-6 trans-signaling is independent of neutrophils. (A) Neutrophils were depleted with the anti-Ly6G/Ly6C monoclonal antibody before CCl_4 injection. Liver damage was quantified via serum ALT levels 24 h post CCl_4 treatment. ALT values were significantly reduced in the sera of neutrophil depleted animals ($P<0.005$). (B) Quantitative blood flow cytometric analysis of neutrophils in percent at the indicated time points after CCl_4 induced liver damage was carried out in C57Bl/6 N mice treated with CCl_4 after previous injection of sgp130Fc or mock treatment. (C) Quantitative blood flow cytometric analysis of neutrophils in percent at the indicated time points after CCl_4 induced liver damage was performed in C57Bl/6 N and sgp130Fc transgenic mice treated with CCl_4.

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.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbadis.2010.07.023.

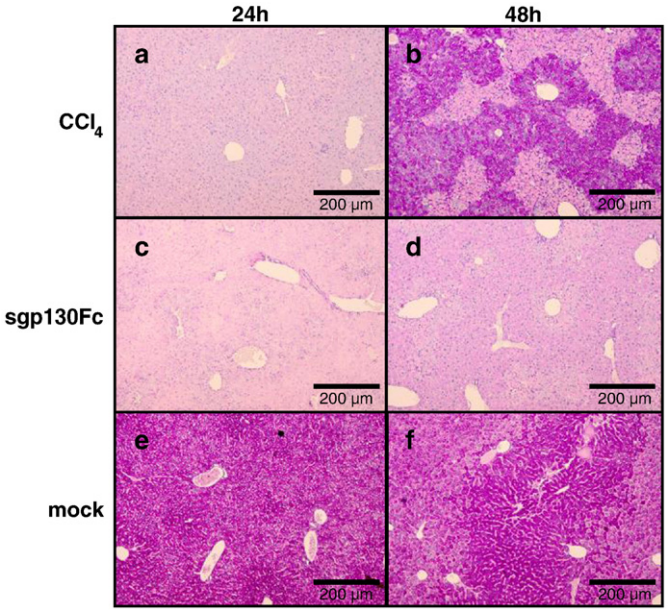

Fig. 6. Glycogen staining in livers upon CCl_4 damage. PAS staining of liver sections 24 and 48 h after CCl_4 induced liver damage showed decelerated glycogen restoration in sgp130Fc pretreated animals 48 h after CCl_4 injection when compared to mice treated with CCl_4 only. (a) CCl_4 only 24 h and (b) CCl_4 only 48 h post CCl_4. (c) sgp130Fc pretreated 24 h and (d) sgp130Fc pretreated 48 h post CCl_4. (e) Mock (oil only) treated for 24 h and (f) 48 h.

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