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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