Impact of IL-6 classic- and IL-6 trans-signaling on Concanavalin A immune-mediated liver damage

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1 Introduction

1.1 The Liver

The liver is the biggest glandular organ in the vertebrate body and is divided into four lobes of unsymmetrical shape and size. Blood is carried to the liver by the hepatic artery and the portal vein. The hepatic artery carries oxygen-rich blood from the aorta. Blood from the digestive system is first filtered by the liver before it migrates to the rest of the body. The portal vein carries blood containing digested food from the small intestine. These blood vessels subdivide in the liver repeatedly, terminating in very small capillaries. Each capillary leads to a lobule in which it branches out to even smaller vessels called sinusoids. The liver is composed of thousands of lobules, and each lobule consists of hepatocytes, the parenchymal and metabolic cells of the liver (1).

The liver has a wide range of functions. It plays a major role in metabolism including controlling the blood levels of lipids, amino acids and glucose. The liver converts carbohydrates into glucose and *vice versa* glucose into its storable form glycogen to provide persistant energy supply for the body. When the blood sugar level declines, glycogen is metabolized back into glucose. Moreover, the liver generates bile, an alkaline compound which is stored in the gall bladder and assists in digestion by emulsification of lipids (2). The liver decomposes ammonia, a toxic waste product of protein metabolism, into urea, which is then discarded via the urine. Furthermore, degradation of erythrocytes, plasma protein synthesis, hormone production, detoxification and production of biochemicals necessary for processing digested food are key processes taking place in the liver. It also has an intrinsic storage function for iron, vitamins and other essential chemicals. Besides fighting infections in the body by purging the blood of particles and microorganisms including bacteria, the liver is also a site for induction of immune-tolerance due to its exposure to various compounds of nutrition every day (3).

The liver is the only internal human organ capable of natural regeneration and mass recovery after tissue loss, since only 25% of the liver can regenerate into a whole liver. This regeneration process is primarily accomplished by hepatocytes

re-entering the cell cycle and liver resident stem cells, called hepatic oval cells, which can differentiate into hepatocytes (1,2).

1.2 Liver diseases

Liver diseases are categorized by the cause as well as the effect which they have on the liver. Causes may include infection, injury, exposure to drugs or toxic compounds, an autoimmune process or a genetic defect (4). These causes can lead to hepatitis, cirrhosis, fatty liver and potentially to liver cancer (5). Liver diseases show a great diversity of disturbances in liver function and structure with characteristic hallmarks often leading to different forms of hepatitis and cirrhosis (6). Fatty liver is the most common indication of alcohol-induced liver disorders. Fat accumulates inside the liver cells, causing cell enlargement (steatosis) and sometimes cell damage (steatohepatitis), which can lead to cirrhosis (3). Similar changes are also seen in people who do not drink excessive amounts of alcohol but are obese or have diabetes.

Anything that causes severe ongoing injury to the liver can lead to cirrhosis. This is marked by cell death and scar formation and is a progressive disease that creates irreversible damage (7). Cirrhosis is treated only by trying to limit further damage. If it is caused by a virus or another treatable cause of liver injury, treating the cause can stabilize the disease and prevent impairment of liver functions. Cirrhosis has no signs or symptoms in its early stages, but as it progresses, it can cause ascites, muscle wasting, bleeding from the intestine, easy bruising, gynecomastia, and a number of other problems (8). In extreme cases, a liver transplantation may be needed. Since the liver is responsible for the metabolism of alcohol, drugs, and environmental toxins, prolonged exposure to any of these substances can also cause hepatitis and/or cirrhosis and have the potential to cause life-threatening acute liver failure.

Hepatitis can be divided in two major forms. One, in which the liver is inflamed quickly called acute hepatitis, and one, in which the liver is inflamed and damaged slowly over a long period of time called chronic hepatitis. While hepatitis can be caused by any of the means mentioned above, most commonly it is due to infection by one of several viruses, termed hepatitis viruses (8). These viruses

have been named in the order of their discovery as hepatitis A, B, C, D, and E. In addition to the hepatitis viruses, other viruses can also cause hepatitis including Cytomegalovirus, Epstein-Barr Virus, and Yellow Fever Virus (8). Deviant presentation of human leukocyte antigen (HLA) class II on the surface of hepatocytes, possibly due to genetic predisposition or acute liver infection, causes a cell-mediated immune response against the own liver, resulting in autoimmune hepatitis (AIH) (9). AIH can be divided into three subtypes depending on the targets of expressed IgG antibodies (10). Type I is characterized by elevated levels of antinuclear antibody (ANA) and anti-smooth muscle actin antibody (SMA) in the blood. Type II shows high levels of liver/kidney microsomal antibody (LKM-1), whereas anti soluble liver antigen (SLA) is representative for Type III AIH (11).

Another form of hepatitis is ischemic hepatitis, also known as shock liver, which is marked by decreased blood flow (perfusion) to the liver and is usually due to shock or low blood pressure. It can be caused by local problems with the blood vessels that supply oxygen to the liver such as thrombosis, or clotting of the hepatic artery which partially supplies blood to liver cells. Blood testing usually shows high levels of the liver transaminase enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), due to the damaged hepatocytes leaking out these enzymes into the blood steam (3).

Although the pathogenesis of hepatic diseases remains vague, several lines of evidence suggest that cytokines may play a predominant role in the pathogenesis of hepatic diseases. It is well known that several cytokines, especially Tumor Necrosis Factor alpha (TNF-α), show hepatoxicity and are suggested to cause hepatic damage in clinical and experimental sepsis and endotoxemia (12). During endotoxemia, toxins that are intracellular resident and not secreted in a soluble form by life bacteria are released when bacteria cells are lysed. Serum levels of TNF-α, Interleukin-1 (IL-1) and IL-6 were elevated in patients with alcoholic hepatitis (13,14), suggesting an involvement of these cytokines in this liver lesion. With regards to viral hepatitis, it is suggested that T-cell dependent cytotoxicity against virus infected hepatic cells is responsible for the hepatic lesions (15). Cytokines, however, may also be involved in the hepatocyte injury directly or by activating the immune system (16), since cytokines, inluding TNF-α, IL-1, IL-2,

IL-4, and Interferon gamma (IFN-γ), are reported to be elevated in patients with chronic viral liver disease (17-19), and cells expressing Interferon alpha (IFN-α) and IFN-γ are localized in the liver tissue of these patients (20).

When cancer does arise in the liver, it is called primary liver cancer (21). The most common type is the hepatocellular carcinoma, which means cancer that develops in the liver's hepatocyte cells. Hepatitis and cirrhosis are the predominant risk factors to develop hepatocellular carcinoma (22). In some cases cancer starts in other parts of the body and then metastasizes (spreads) to the liver called metastatic liver cancer (23).

1.3 Liver as an immune organ

Structurally and functionally qualification of the liver as a lymphoid organ has been implied in several studies (24-26). The liver can be considered as an immune organ because it hosts the largest population of tissue resident macrophages named Kupffer cells (KCs) in the body and different types of dendritic cells (DCs), and it is also uniquely enriched in T-lymphocytes, natural killer (NK) cells, and natural killer T-cells (NKT) (27,28). Polymorphonuclear leukocytes (PMN), of which neutrophils are predominant, are primarily recruited to the site of injury in the liver (29). With the exception of T-lymphocytes, all of these cells are members of the innate immune system. Blood, enriched of potential antigens coming from the gastrointestinal tract, is constantly passing the network of liver sinusoids, provides contact to the immune cell populations and induces immune responses against pathogens or immunological tolerance towards harmless antigens (26).

In response to inflammatory signals, monocytes and macrophages are rapidly recruited to the liver, and these cells have similar functional profiles as KCs (30). Macrophages and KCs respond to pathogenic factors with production of proinflammatory cytokines and chemokines such as TNF-α, IL-6, Chemokine (C-C motif) ligand 2 (CCL2)/Monocyte chemotactic protein-1 (MCP-1) and Chemokine (C-X-C motif) ligand 1 (CXCL1) (KC) (31), which is the mouse homolog to human IL-8. In addition, also hepatocytes produce KC, Macrophage-Inflammatory Protein-2 (MIP-2) and Cytokine-Induced Neutrophil Chemoattractant-1 (CINC-1), which are all potent attractants for neutrophils (32)

after inflammatory stimulus. Because of the high mobility of neutrophils and their capacity to generate cytotoxic mediators, the main function of neutrophil recruitment to the site of inflammation is to rapidly eliminate invading microorganisms and apoptotic cells (33). Neutrophils are phagocytic leukocytes that represent the first line of defense during infection and injury (34). Activated neutrophils have high levels of myeloperoxidase, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, and inducible nitric oxide synthase expression, resulting in their capacity to exert oxidative stress-mediated tissue damage (35). In the liver, the excessive inflammatory response carries the risk of additional tissue damage as seen during ischemia-reperfusion, acute liver failure, alcoholic liver disease, and viral hepatitis (36). Besides chemokines, adhesion molecules, such as vascular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, play an important role in neutrophil infiltration and contribute to liver injury (37).

NK cells represent a population of lymphocytes with potent cytolytic activity against virus-infected or tumor cells. Hepatic NK cells are regulated by Kupffer-cell derived cytokines, e.g. IL-12 and IL-18, as well as NKT cell-derived IL-4 (38). Hepatic NK cells modulate T-cell responses in the liver, promote intracellular changes in endothelial cells and hepatocytes (mainly via IFN-γ), and can even directly induce hepatocyte death or cell lysis (39). NKT cells are a subpopulation of extraordinary T-cells, as they express surface markers of T- and NK cells, which are found at unusually great quantities in the liver. From studies of experimental liver injury in mice after administration of the plant-derived lectin Concanavalin A (ConA), NKT cells have been determined as crucial factors promoting acute liver damage by release of IL-4, IFN-γ and direct induction of Fas-mediated hepatocyte apoptosis (40).

1.4 The multifunctional cytokine IL-6

Interleukin-6 (IL-6) is a pleiotropic cytokine with a size of 20-28 kDa depending on its glycosylation state (41). Primary it was found to be a B-cell differentiation factor that induces the production of immunoglobulins (42). Nowadays IL-6 is known to be crucial in regulating diverse biological activities and immune modulating processes. IL-6 is secreted by lymphoid cells, e.g. T-cells, B-cells, macrophages

and monocytes (43). It induces growth of T-cells and differentiation of cytotoxic T-cells by enhancing the expression of IL-2 and IL-2 receptor (44). Furthermore, IL-6 was demonstrated to induce differentiation of macrophages (45) and megakaryocytes (46). Besides its expression by lymphoid cells, IL-6 is also produced by various types of nonlymphoid cells, e.g. fibroblasts, keratinocytes, endothelial cells, adipocytes and several tumor cells (43).

Considering its extensive ability to activate host immune responses after infection, IL-6 expression has to be stringently regulated and the cytokine is expressed in undetectable or very low levels under normal physiological conditions (47). It is usually secreted at local tissue sites and is released in almost all situations of homeostatic perturbation including acute infection, trauma and endotoxemia. IL-6 can also become chronically elevated, resulting in the prolonged activation of its biological activities. A dysregulation in IL-6 values can reverse the primarily helpful responses into harmful conditions and has been shown to be relevant in patients with colon carcinoma (48,49). Moreover, IL-6 has been identified as a growth factor for renal cell carcinoma cells (50) and multiple myeloma cells (51). Capable of acting in an autocrine, paracrine, and endocrine manner, IL-6 is a pivotal mediator of fever (52) and regulator of hepatic acute phase response by stimulating hepatocytes to produce C-reactive protein, fibrinogen and serum amyloid A (53).

IL-6 is a four α-helix bundle protein belonging to the family of gp130 cytokines including interleukin-11 (IL-11), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotropin-1 (CT-1), cardiotrophin-like cytokine/neurotrophin-1/B-cell stimulating factor 3 (CLC/NNT-1/BSF-3), new neuropoietin (NPN), IL-27 and IL-31 (54-56). IL-6 first binds its membrane-bound receptor, the IL-6 receptor (IL-6R), which is not involved in signal transduction. Thereupon, the IL-6/IL-6R complex sequentially associates with two molecules of gp130. Dimerization of gp130 leads to intracellular signaling events like activation of JAK (Janus kinase) tyrosine kinase family members, followed by the activation of transcription factors of the STAT (signal transducers and activators of transcription) family and their subsequent transport into the nucleus. Another major signaling pathway for IL-6 is the Mitogen-Activated Protein Kinase (MAPK) cascade (57).

IL-6 knockout mice have been shown to develop normally but exhibit a massively suppressed acute phase response after tissue damage or infection. They suffer from impaired liver regeneration, which could be reversed by IL-6 treatment (58). IL-6 in the liver is mainly secreted by Kupffer cells and induces paracrine activation of hepatocytes after TNF-α stimulation (59). IL-6 deficient mice are impaired in controlling infection with facultative intracellular bacteria like *Listeria monocytogenes* and show compromised T-cell dependent antibody responses (60). Furthermore, IL-6 knockout mice are completely protected from collagen-induced arthritis, a mouse model for rheumatoid arthritis (61) and they are resistant to experimental autoimmune encephalomyelitis (EAE) induced by immunization with myelin oligodendrocyte glycoprotein (MOG), an animal model for the demyelinating disease multiple sclerosis (62). Also cancer development (63), colitis (65,79), peritonitis and acute inflammation (66) are accompanied by altered IL-6 signaling. All in all, the phenotype of IL-6 knockout mice in diverse disease models underlines that IL-6 is an important cytokine involved in regulation of host response to infection, and that dysregulation of IL-6 contributes to several pathological conditions in various diseases, e.g. rheumatoid arthritis, multiple sclerosis and liver injury.

1.5 IL-6 trans-signaling and soluble gp130

Many cytokine receptors exist in a soluble form consisting of the extracellular part of their corresponding transmembrane receptor. Most soluble cytokine receptors have antagonistic properties by competing with their membrane-bound counterparts for the matching ligands, e.g. IL-1R, TNFR and IL-4R (67-69). In contrast, soluble IL-6R (sIL-6R) acts as an agonist of IL-6 activity. While gp130 is found on all cell types, expression of the membrane-bound IL-6R is largely confined to hepatocytes, neutrophils, macrophages and some lymphocyte subpopulations (70). Two mechanisms of IL-6 signaling are described. Binding of IL-6 to the membrane bound IL-6R induces the recruitment of two gp130 molecules and the downstream activation of intracellular signalling cascades via gp130, a process termed classic IL-6 signaling. Therefore, cells lacking the membrane-bound IL-6R are not able to respond to classic IL-6 signaling. This lack of cell responsivness to IL-6 can be overcome by a process called IL-6 trans-signaling. Herein, a naturally occuring soluble form of the IL-6R (sIL-6R) is found

in body fluids of healthy humans (25-35 ng/ml in serum), which is either produced by proteolytic cleavage or alternative splicing (71,72). In humans, induced cleavage of the IL-6R is mediated by the metalloprotease Tumor Necrosis Factor-alpha Converting Enzyme (TACE/ A Disintegrin And Metalloproteinase ADAM17), whereas constitutive shedding is dependent on ADAM10 (73). During IL-6 trans-signaling, sIL-6R binds to IL-6 in solution with the same affinity as membrane-expressed IL-6R and the complex of IL-6 and sIL-6R can stimulate cells lacking membrane-bound IL-6R by activating gp130. Due to the ubiquitous expression of gp130, the alternative IL-6 signaling pathway has important biological consequences since it drastically enlarges the spectrum of IL-6 target cells and can stimulate nearly all cells of the body. Besides, IL-6 trans-signaling has been shown to affect cells that already express membrane bound IL-6R. In the liver, hepatocytes have been demonstrated to express far more gp130 than IL-6R and the total number of activated gp130 molecules is increased when IL-6 and sIL-6R are present (74).

In addition to the IL-6R the second component of the IL-6 receptor complex, gp130, also exists in a soluble form (sgp130). Relatively high circulating levels of sgp130 are detected in human blood (100-400 ng/ml) and sgp130 may associate with the IL-6/sIL-6R complex to inhibit signaling via membrane-bound gp130 (75-78). It has been postulated that sgp130 is the natural inhibitor of IL-6-transsignaling and that sgp130 acts as a buffer to prevent the stimulation of almost all cells in the body by the IL-6/sIL-6R complex (79). Alternative splicing has been implicated as the predominant mechanism in the cellular release of sgp130 (80). A designer protein, termed sgp130Fc, was constructed by fusing the complete extracellular region of gp130 with the Fc-part of a human IgG-antibody. The resulting protein is a preformed dimer and therefore strongly resembles the receptor conformation on the living cell. It has been shown that dimerized sgp130Fc is able to inhibit IL-6/sIL-6R-mediated responses with a 10-fold higher activity than monomeric sgp130 (79). Therefore, the application of sgp130Fc selectively blocks IL-6 trans-signaling without affecting classic IL-6 signaling and enables to distinguish between those mechanisms of signaling. So far, application of sgp130Fc has been shown to have beneficial effects in animal models of inflammation (65), autoimmunity (81) and cancer (82) pointing to the relevance of

IL-6 trans-signaling and the role of sgp130Fc as a powerful tool in medical treatment.

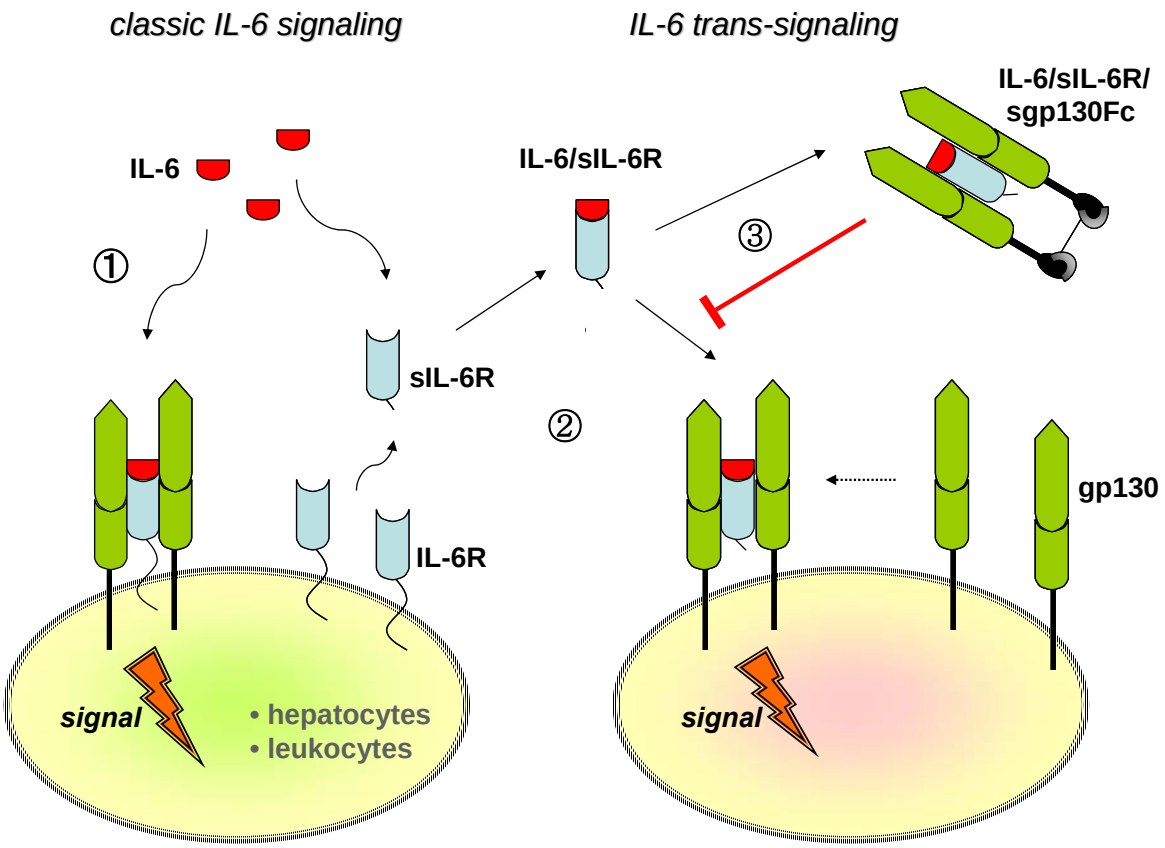

Figure 1: Schematic illustration of classic IL-6 signaling and IL-6 trans-signaling. ① Cells that express both gp130 and the IL-6R are responsive to IL-6 (classic signaling). ② Cells that express only gp130 can be activated by the complex of IL-6/sIL-6R. Activation of such cells is termed trans-signaling. ③ Molar excess of sgp130Fc competitive inhibits IL-6 trans-signaling by binding IL-6/sIL-6R complexes without affecting classic IL-6 signaling.

1.6 Immune related liver damage induced by Concanavalin A

The activation of T-lymphocytes occurs as initial step in the pathophysiology of human autoimmune liver diseases (83) or viral hepatitis (84). T-cell mediated hepatitis in mice can be induced by injection of the T-cell mitogenic plant lectin Concanavalin A (ConA), a protein originally extracted from the jack bean (*Canavalia ensiformis*). Tissue injury caused by intravenous ConA administration

is limited to the liver (85). When treated with ConA, mice develop an acute, partly apoptotic, hepatic injury that thereafter is displaced by massive necrosis (85,86). Acute liver failure induced by intravenous injection of ConA is characterized by strong leukocyte infiltration to the liver that occurs with a marked increase in levels of alanine aminotransferase (ALT) and asparagine aminotransferase (AST) in the blood shortly (8-24h) after ConA challenge (85,87-89). The cellular and molecular mechanisms based on ConA-induced hepatitis have been examined in great detail, but are still not fully understood. Although $CD4^+$ T-cells have been shown to be key players in this model (85,90), there are several cell depletion studies showing that NKT cells (89), Kupffer cells (91) and neutrophils (87) are required to induce ConA-mediated liver injury. Immunregulatory cytokines play a central role in the pathogenesis of ConA-induced hepatitis with studies pointing to a proinflammatory role of TNF-α (90), IFN-γ (92), IL-12 and IL-4 (93), and a protective role of IL-10 (94) and MCP-1 (95).

Injection of ConA into mice leads to an increased release of IL-6 (85,90), but the effects of IL-6 in this model are ambiguous. IL-6 deficient mice show a higher susceptibility towards ConA-induced liver injury compared to wild-type animals (92). Studies with anti-IL-6 antibodies reveal a more complex situation, depending on the time of administration. Antibody injection shortly (2.5 h) after ConA leads to delayed liver injury, whereas injections 24 h and 1 h before ConA result in increased liver damage (96). In addition, injections of recombinant IL-6 24 - 3 h before ConA injection completely prevented liver damage, whereas IL-6 administration 3 - 6 h after ConA challenge resulted in enhanced liver injury (90). In consequence, endogenous IL-6 present before ConA administration seems to be hepatoprotective, while IL-6 production after ConA injection promotes liver damage. Furthermore, IL-6 trans-signaling has been shown to have an impact on neutrophil recruitment in a murine model of peritoneal inflammation. In this model, reconstitution of IL-6 signaling in IL-6 deficient mice with IL-6 and its soluble receptor corrected imbalance of neutrophil-activating CXC chemokine expression and suppressed neutrophil infiltration (97). In addition, neutrophil-depleted mice show reduced $CD4^+$ T-cell recruitment to the liver and diminished liver injury after ConA administration (87).

However, apart from the phenotypical effects of IL-6 during ConA-induced hepatitis, less is known about the molecular basis. Some data indicate that the protective effect may be due to IL-6 induced STAT3 activation and subsequent suppression of IFN-γ signaling and induction of the anti-apoptotic gene B-Cell Lymphoma-Extra Large (Bcl-X_L) (92). The harmful effect of late IL-6 application has been hypothesized to be due to stimulated expression of TNF-receptors on hepatocytes and thereby promoting the harmful role of TNF-α in ConA-hepatitis (98).

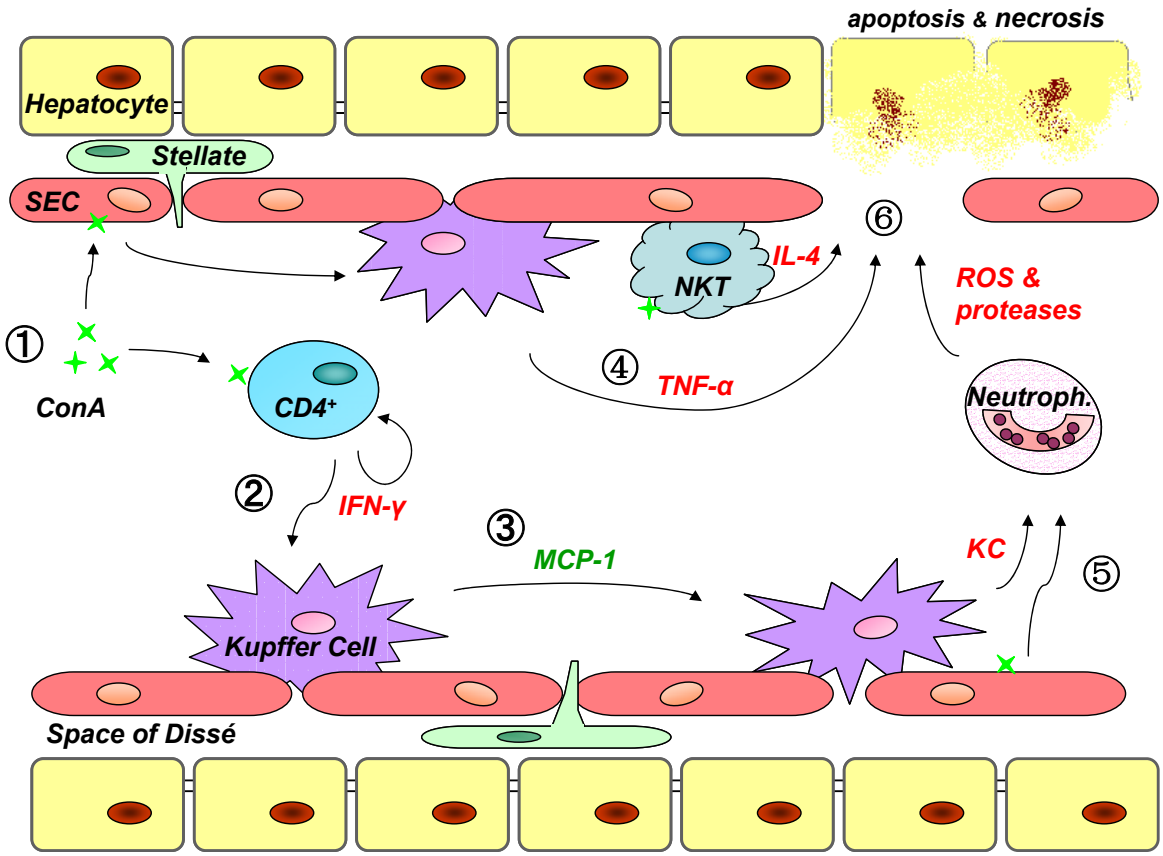

Figure 2: Suggested mechanisms of Concanavalin A induced signaling and cell activation in the liver. ① Upon intravenous injection to mice, ConA binds to sinusoidal endothelial cells (SEC) and activates $CD4^+$ T-cells as well as NKT cells. ② These cells activate Kupffer cells and produce IFN-γ which seems to be important for activation of intrahepatic $CD4^+$ T-cells and NKT cells. ③ MCP-1 release from Kupffer cells recruits further Kupffer cells/macrophages. ④ The Kupffer cells produce TNF-α that might induce apoptosis of SEC and hepatocytes. Moreover, neutrophils, in dependence of Kupffer cell- and SEC-derived KC are recruited into hepatic parenchyma ⑤ and seem to mediate liver cell necrosis together with NKT cells by secretion of reactive oxygen species (ROS), proteases and IL-4 ⑥.

1.7 Aim of this work

A wide range of studies have shown that immune cells, cytokines and cytokine dependent pathways have an impact on the development of liver failure, chronic liver disease and hepatic inflammation (31,83,84). A dysregulation in cytokine interaction after liver injury can lead to massive apoptosis and necrosis which are pivotal results in various acute and chronic liver diseases, e.g., viral and autoimmune hepatitis (99,100). IL-6 pathways revealed protective functions in many experimental liver disease models (101,102) and therefore are an attractive target for a pharmacological intervention in acute and chronic liver diseases. Injection of ConA into mice mimics histological and pathological hallmarks of autoimmune and T-cell mediated hepatitis. In the present work, the role of IL-6 classic- and IL-6 trans-signaling in ConA-induced hepatitis was investigated to further elucidate the complex effects of IL-6 signaling in immune related liver injury and in order to provide new therapeutic approaches. Since it is known that a high dose of ConA rapidly leads to symptoms of acute hepatitis or even death, a more moderate dose was used in this work to simulate the initial processes of liver-inflammation and -injury and to analyze the influence of IL-6 signaling within the initial phase of processes in more detail. Furthermore, IL-6 has been shown to have dual functions with contrary effects in this model (90,96) and several types of immune cells have been shown to be necessary for the onset of liver inflammation in this model, but a connection between one immune cell type and IL-6 signaling was not made. Immune cell candidates were examined in different approaches of manipulated IL-6/gp130 signaling in ConA treated mice. Moreover, the relevance of IL-6 trans-signaling in ConA pathology was examined by using the designer protein sgp130Fc for *in vivo* experiments. Besides the relevance of IL-6 signaling in immune cell recruitment and initial processes in the ConA model, also the role of IL-6 signaling in tolerance induction towards a re-challenge with ConA was examined by using IL-6 deficient mice.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used were obtained from CarlRoth (Karlsruhe, Germany), Sigma-Aldrich (Deisenhof, Germany) or Merck (Darmstadt, Germany), unless otherwise stated. If not mentioned otherwise deionized water (Millipore) was used as solvent.

2.1.2 Animals

C57BL/6N and IL-6 deficient mice with C57BL/6N background were purchased from Charles River (Sulzfeld, Germany) or bred by mating in the animal facility. All animals were maintained at a 12-hour light-dark cycle under standard conditions and provided with food and water *ad libitum*. For all experiments 3 – 10 mice per group were used.

2.1.3 Animal food

The standard diet was obtained from SSNIFF Spezialdiäten GmbH (Soest, Germany).

Standard :	crude protein 19 %
	crude fat 3.30 %
	crude fibre 4.90 %
	crude ash 6.70 %
	Additives: Vitamin A: 15.000 IE/kg; Vitamin D3: 1.000 IE/kg; Vitamin E: 100 mg/kg; Cu: 5 mg/kg

2.1.4 Recombinant proteins

Concanavalin A (ConA)	Concanavalin A, Type IV, 100 mg (Cat.No.: C2010, Sigma-Aldrich, Germany)
human interleukin-6 (huIL-6)	prepared and prurified as described in (139)
soluble gp130Fc (sgp130Fc)	purified from supernatant of stably transfected CHO-cells as described in (79)

2.1.5 Solutions and Buffers

Blocking buffer *(Western blot)*:	5 % milk powder or BSA in TBS-T
Blotting buffer *(Western blot)*:	25 mM Tris-HCl, pH 8.3 192 mM glycine 20 % (v/v) methanol
Destaining solution *(protein gels)*:	10 % (v/v) acetic acid 40 % (v/v) methanol
Dulbecco's PBS (1x) *(ConA solutions)*:	Cat.: H15-002 (w/o) Ca & Mg; PAA Laboratories, Pasching,Austria
EDTA buffer *(Blood preparation)*:	2 mM EDTA 0.2 % (w/v) BSA in PBS
Erylysis buffer *(T cell isolation)*:	150 mM NH_4Cl 10 mM $KHCO_3$ 100 nM EDTA
FACS buffer *(FACS)*:	1 % (w/v) BSA in PBS 0.05 % (w/v) NaN_3
FACS lysing solution *(Blood preparation)*:	Cat.: 349292 BD Bioscience, Heidelberg, Germany
Histopaque 1077 *(T cell isolation)*:	Cat.: 10771, 100 ml; Sigma-Aldrich, Deisenhof, Germany
Lysis buffer *(cell lysis)*:	150 mM NaCl 2 mM EDTA 50 mM Tris-HCl, pH 7.4 1 % (w/v) Triton X-100 1 % (w/v) NP-40 1 mM Na_3VO_4

	1 mM NaF
	1 x protease inhibitors
Phosphate buffered saline *(PBS)*:	150 mM NaCl
	8 mM Na_2HPO_4, pH 7.4
	1.7 mM NaH_2PO_4, pH 7.4
PBS-T:	0.05 % (v/v) Tween-20 in PBS
Protease-inhibitors:	COMPLETETM pills, Roche (Mannheim, Germany)
	Resuspending 1 pill in 50 ml Lysis buffer
RPMI medium *(T cell isolation)*:	RPMI 1640, Cat.: E15-039
	PAA Laboratories, Pasching, Austria
Running gel 10 % (15 %) *(protein gels)*:	7.74 ml (4.5 ml) deionized water
	5.1 ml (5.1 ml) 1.5 M Tris, pH 8.8
	0.2 ml (0.2 ml) 10 % SDS
	6.6 ml (9.9 ml) 30 % Acrylamide-Bis
	0.2 ml (0.2 ml) 10 % APS
	0.02 ml (0.02 ml) TEMED
Sample-buffer (5x) *(protein-gels)*:	10 % (w/v) SDS
	5 % (w/v) β-mercaptoethanol
	50 % (w/v) glycerol
	0.13 % (w/v) bromphenol blue
	300 mM Tris-HCl, pH 6.8
Sample-buffer (2x) *(protein-gels)*:	4 % (w/v) SDS
	20 % (w/v) glycerol
	5 % (v/v) β-mercaptoethanol
	0.13 % (w/v) bromphenol blue
	125 mM Tris-HCl, pH 6.8

SDS running buffer *(protein-gels)*:	25 mM Tris-HCl, pH 8.3 192 mM glycine 0.1 % (w/v) SDS
Stacking gel (4 %) *(protein gels)*:	3.72 ml deionized water 0.625 ml 0.5 M Tris-HCl, pH 6.8 0.05 ml 10 % SDS 0.67 ml 30 % Acrylamid-Bis 29:1 25 µl 10 % APS 7 µl TEMED
Coomassie staining *(protein gels)*:	40 % (v/v) ethanol 10 % (v/v) acetic acid 0.1 % (w/v) coomassie R250
Stripping solution *(Western blot)*:	0.5 M NaCl 0.5 M acetic acid
TBE (0.5x) *(agarose-gels)*:	44.5 mM boric-acid 10 mM EDTA, pH 8.0 44.5 mM Tris-HCl
Thiobarbituric acid *(TBA)*:	67 mg in 1 ml DMSO + 9 ml H_2O
1,1,3,3-tetramethoxy propane *(TMP)*:	4.167 µl in 1 ml EtOH + 49 ml H_2O (500 µM)
Trichloroacetic acid	10 % (w/v) in H_2O
Tris buffered saline *(TBS)*:	10 mM Tris-HCl, pH 8.0 150 mM NaCl
TBS-T:	1 % (v/v) Tween-20 in TBS

2.1.6 Primary Antibodies

anti-β-actin:	rabbit polyclonal antibody raised against the endogenous ß-actin (Cell signaling, Boston, USA) IB: dilution 1:1.000 (5 % BSA in TBS-T)
anti-Phospho-STAT3:	rabbit monoclonal antibody which detects endogenous levels of STAT3 only when phosphorylated at tyrosine 705 (Cell signaling, Boston, USA) IB: dilution 1:2.000 (5 % BSA in TBS-T)
anti-STAT3:	mouse monoclonal antibody which detects endogenous levels of total STAT3 protein (Cell Signaling) IB: dilution 1:1.000 (5 % milk powder in TBS-T)
anti-Ly6G/Ly6C	Rat monoclonal antibody rat monoclonal antibody which reacts with a common epitope on Ly-6G and Ly-6C, previously known as the myeloid differentiation antigen Gr-1. The antibody recognizes granulocytes (neutrophils and eosinophils) and monocytes. (BD Biosciences)

2.1.7 Secondary antibodies

All horseradish-peroxidase coupled secondary antibodies were purchased from Amersham Bioscience (Buckinghamshire, United Kingdom) and used in a dilution of 1:5.000.

2.1.8 FACS antibodies

BD Bioscience, Heidelberg, Germany

CD11b:	PE rat anti-mouse CD11b (Cat.: 553311)
CD3e:	APC-hamster anti-mouse CD3e (Cat.: 553066)
Ly6GC:	FITC anti-mouse Ly6GC (Cat.: 553126)
NK1.1:	PE mouse anti-mouse NK1.1 (Cat.: 557391)
CD69:	PE-Cy7 hamster anti-mouse (Cat.: 552879)

eBioscience, NatuTec GmbH, Frankfurt, Germany

CD4:	FITC anti-mouse (Cat.: 11-0042-85)

2.1.9 Kits

Greiner Kits for detection of transaminases

(Greiner Diagnostic, Bahlingen, Germany):

GPT (ALAT) IFCC	Cat.:142000 (5x20/2x10 ml)	for detection of alanine aminotransferase
GOT (ASAT) IFCC	Cat.:141000 (5x20/2x10 ml)	for detection of aspartate aminotransferase

R&D - DuoSet ELISA Development System

(R&D Systems, Wiesbaden, Germany):

human sgp130	Cat.: DY228
murine IL-6	Cat.: DY406

murine IL-4	Cat.: DY404
KC	Cat.: DY453
MCP-1 (JE)	Cat.: DY479
murine TNF-α	Cat.: DY410

2.1.10 Electric devices

FACSCanto BD Bioscience

TECAN Rainbow 96-well UV/ELISA Reader (Railsheim, Germany)

Olympus BX50 microscope and Olympus UC30 camera system (Tokyo, Japan)

BioRad Power Pac 300 & 200

BioRad Transblot SD (SemiDry) transfer cell

BioRad Mini Trans-Blot cell

Eppendorf Centrifuge 5415R (Heraeus Biofuge pico, Kendro Laboratory)

Eppendorf Thermomixer 5436

Ultrospec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech)

Jouan CR3i cooling centrifuge

2.1.11 Consumables

Disposable hydrodermic needle 100 Sterican 27 G & 21 G (Braun)

BD Microlane 3 Needles 30 G

Injekt-F 1 ml, single-use fine dosage syringe 2-piece (Braun)

Nunc-Immuno Plate F96 Cert. Maxisorp, Roskilde, Denmark

HistoBond - 76x26x1 mm Microscope Slides (Marienfeld, Lauda-Königshafen, Germany)

Sarstedt, Nümbrecht, Germany

Petri dish 92x16 mm with cams

Microvette Tube CB300 CH

Micro-Test Plate 96 Well

Tube, 15 ml & 50 ml

Pipette tip 1000 µl blue, 200 µl yellow, 10 µl neutral

Safe seal microtube, 2 ml

Micro tube, 1.5 ml

Flow cytometry tubes, 5 ml

2.1.12 Software

Microsoft Word (2000)

Microsoft Excel (2000)

GraphPad Prism 4 (Data editing)

FACSDiva software, BD Bioscience (Flowcytometry detection)

FCS Express V3 (Flowcytometry analysis)

2.2 Methods

2.2.1 Animal treatment

2.2.1.1 Animal husbandry

Procedures involving animals and their care were conducted in conformity with national and international laws according to home office approved project license V 31272241.121-3 (41-3/06). Mice were kept at a 12-hour light-dark cycle under standard conditions and provided with food and water *ad libitum*. All mice were maintained under barrier conditions and were pathogen free as assessed by regular microbiological screening. The animals were kept at 21°C ± 2°C and 60 % ± 5 % humidity in individually ventilated cages (IVC).

2.2.1.2 Intravenous Concanavalin A (ConA) injection

A stock solution with ConA (Cat.No.: C2010, Sigma, Deisenhofen, Germany) of 10 µg/µl was diluted in sterile PBS and stored at -80°C in 1 ml aliquots until used for injections. Before treatment, mice were weighed, hold in a fixing-tube and mice tails were exposed to infrared light for one minute. Liver damage was induced by intravenous injection of ConA dissolved in 100 µl PBS immediately before treatment and applied as one dose of 12.5 or 20 µg/g body weight. Intravenous injection was performed using a 1 ml syringe in combination with a 30 gauge needle (0.3 mm x 13 mm).

2.2.1.3 IL-6 treatment

Mice were intravenously injected with 2 µg IL-6, 6-3 hours before ConA challenge for protection studies or treated with a single injection of IL-6 and sacrificed 4 h later for investigation of peripheral blood neutrophil level.

2.2.1.4 Intraperitoneal injection

200 µl sterile PBS or 200 µl sterile PBS containing antibodies (see 3.2.1.5 & 3.2.1.6) were injected into the peritoneal cavity of mice without anesthesia. Solutions were prepared and loaded under a sterile fume hood. Intraperitoneal

injection was performed using a 1 ml syringe in combination with a 27 gauge needle.

2.2.1.5 IL-6 antibody and sgp130Fc treatment

Total IL-6 signaling was blocked with a single injection of purified rat anti-mouse MP5-20F3 IL-6 antibody (BD Bioscience, Heidelberg, Germany). Mice were intraperitoneally injected with 200 µg antibody 16 h prior to ConA injection. IL-6 trans-signaling was blocked with a single injection of purified sgp130Fc. Mice were injected i.p. with 250 µg sgp130Fc 16 h prior to ConA injection.

2.2.1.6 Neutrophil depletion

Neutrophils were depleted using purified rat anti-mouse Ly6G/Ly6C antibody (BD Bioscience, Heidelberg, Germany). Mice were intraperitoneally injected with 100 µg antibody 18 h prior to ConA challenge. Depletion was controlled with stainings for neutrophils on paraffin tissue sections.

2.2.2 Serum aminotransferase measurements

Serum alanine aminotransferase (ALT) was determined using the ALT detection kit (Greiner Diagnostics, Bahlingen, Germany) according to manufacturer's instructions. For measurements of aminotransferas, serum was diluted 1:30 in PBS to stay within detection kit threshold value conditions.

2.2.3 Enzyme-linked Immunosorbent Assay (ELISA)

Cytokine levels were measured via ELISA using murine DuoSet ELISA Kits (R&D Systems, Wiesbaden, Germany) according to manufacturer´s instructions. Standard and serum samples were diluted with 1 % BSA/PBS. For IL-6 ELISA (DY406), serum was diluted 1:20 in 1 % BSA/PBS. For MCP-1 (DY479) and KC ELISA (DY453), serum was diluted 1:60 in 1 % BSA/PBS. Control detection of sgp130Fc was performed by using the human sgp130 DuoSet ELISA Kit (DY228, R&D Systems). For the human sgp130 ELISA, samples were diluted 1:2000 in 1 % BSA/PBS and recombinant sgp130Fc was used as a standard with the highest concentration being 10 ng/ml. For murine liver IL-4 (DY404) and murine

TNF-α ELISA (DY410) analysis, liver lysates from homogenized liver tissue were used at a 10 µg/µl concentration diluted in 1 % BSA/PBS.

2.2.4 Fluorescence-activated cell sorting (FACS)

2.2.4.1 Flow cytometry analysis of peripheral blood granulocytes (PMNs)

25 µl whole blood was given to 100 µl EDTA-buffer (2 mM EDTA, 0.2 % BSA in PBS) and immediately processed for flow cytometry stainings. Samples were blocked with 0.5 µl rat-serum (Jackson ImmunoResearch, Suffolk, England) and 0.5 µl rat anti-mouse Fc block (BD Bioscience, Heidelberg, Germany) on ice for 5 min. Whole blood cells were stained in the dark with fluorochrome directly conjugated mAbs: PE rat anti-mouse CD11b (BD Bioscience, Heidelberg, Germany, Cat.: 553311), APC-hamster anti-mouse CD3e (BD Bioscience, Cat.: 553066) and FITC anti-mouse Ly6GC (BD Bioscience, Cat.: 553126).

After 30 min of incubation at 4°C, red blood cells were lysed with the FACS lysing solution (BD Bioscience, Cat.: 349292) for 10 min at room temperature. The cells were washed and resuspended in FACS buffer (PBS, 0.2 % BSA). Stained samples were analyzed with a fluorescence-activated cell sorter scan (FACScanto) cytometer (BD) and processed by FACSDiva software (BD).

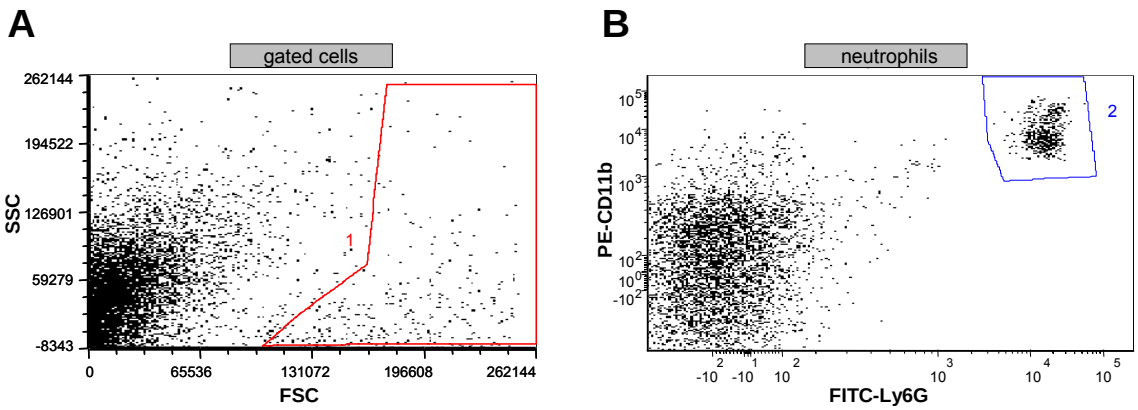

Figure 3: Exemplary evaluation of peripheral blood neutrophils by flow cytometry. (A) Forward scatter/side scatter (FSC/SSC) plot showing the cellular distribution in prepared blood samples 8 h after ConA treatment (one representative C57BL/6 animal). Gate 1 was set to exclude both cellular debris and remaining erythrocytes. (B) Gate 2 quantified CD11b/Ly6G double positive cells (neutrophils) from cells selected in gate 1.

2.2.4.2 Liver-derived lymphocyte isolation and analysis by flow cytometry

Livers were excised and pushed through a nylon mash (BD Bioscience, 40 µm cell strainer) in a petri dish containing 10 ml RPMI-1640 medium (PAA, Cat.: E15-039). The cell suspension was centrifuged at 300 g for 5 min and the supernatant was discarded. To get rid of erythrocytes, the cell pellet was resuspended in FACS lysing solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 nM EDTA) for 10 min at room temperature and the supernatant was discarded after centrifugation.

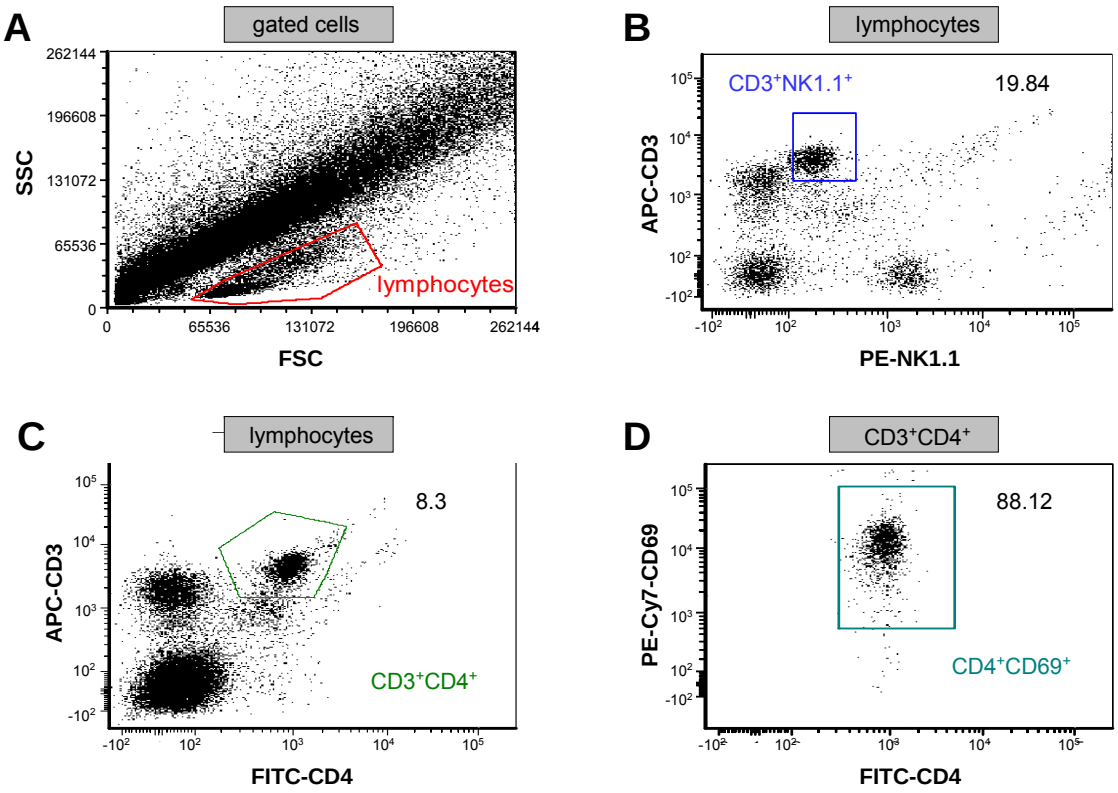

Figure 4: Exemplary evaluation of T-cell populations by flow cytometry. (A) Forward scatter/side scatter (FSC/SSC) plot showing the cellular distribution in prepared liver samples 8 h after ConA treatment (one representative C57BL/6 animal). Lymphocyte gate (A) was set to exclude both cellular debris and remaining erythrocytes. (B) Gated CD3/NK1.1 double positive cells from the lymphocyte gate. (C) Gated CD3/CD4 double positive cells from the lymphocyte gate. (D) Gated CD4/CD69 double positive cells from CD3/CD4 gated cells.

The cell pellet was resuspended in RPMI-1640 medium. 2 ml cell suspension was then overlaid onto 2.5 ml Histopaque (Sigma, Deisenhofen, Germany) and centrifuged for 30 min at 300 g. MNCs were collected from the interface, washed

in 3 ml 5 % FCS/PBS and resuspended in 50 µl 5 % FCS/PBS. Cell populations were stained by addition of appropriate antibodies to the suspension for 30 min at 4°C in the dark, washed and resuspended in 500 µl 2 % FCS/PBS for flow cytometric analysis. NKT cell numbers were determined by using PE mouse anti-mouse NK1.1 (BD Bioscience, Cat.: 557391) and APC-hamster anti-mouse CD3e (BD Bioscience, Cat.: 553066) antibodies. $CD4^+$ T-cell activation was assessed by FITC anti-mouse CD4 (eBioscience, Cat.11-0042-85) and PE-Cy7 hamster anti-mouse CD69 (BD Bioscience, Cat.: 552879) antibodies.

2.2.5 Tissue processing and Immunohistochemistry

2.2.5.1 Tissue processing and embedding

Liver tissue was washed in PBS, transferred into a tissue cassette and fixed in 4 % formaline for 24 h at 4°C. After fixation, tissues were transferred to 70 % ethanol and cassettes were put through a series of graded EtOH baths to dehydrate the tissues. Subsequently the cassettes were transferred to xylene and thereafter to a hot paraffin bath:

- 70 % Ethanol 60 min (x1)
- 95 % Ethanol 45 min (x2)
- 100 % Ethanol 45 min (x2)
- Xylene 60 min (x2)
- Paraffin (65°C) 30 min. (x1)
- Paraffin (65°C) over night (x1)
- Paraffin (65°C) 90 min (x1)

Tissues processed into paraffin were embedded by placing the entire cassette on a heat block. Also warm metal block molds were put on the hot plate. The paraffinized specimens were placed with the cut surface down towards the bottom of the mold. Hot paraffin was added to the mold from a paraffin pot. When the tissue was in the desired orientation the labeled tissue cassette was added on top of the mold as a backing. Subsequently the mold was taken off the hot plate and transferred onto a cold aluminum plate in a freezer. When the wax was completely cooled and hardened (~5 min) the paraffin block was popped out of the mold.

2.2.5.2 Sectioning tissues

Blocks to be sectioned were placed face down on ice for 10 min. The block was inserted into the microtome chuck so the wax block faced the blade and was aligned in the vertical plane. 5 µm sections were cut and picked up with forceps or a fine paint brush and floated on the surface of the 37ºC water bath. Afterwards, the sections were floated onto the surface of clean glass slides.

2.2.5.3 Hematoxylin & Eosin staining

Tissue sections were shortly incubated in Gill3 Hematoxylin (Thermo Scientific, Cheshire, UK), differentiated in 0.5 % acetic acid, rinsed in water and stained with Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany).

2.2.5.4 Neutrophil and pSTAT3 staining

Staining for neutrophils was carried out using a monoclonal rat anti-mouse neutrophil antibody (AbDserotec, Düsseldorf, Germany) diluted 1:2000 in sample diluent (Dako, Glostrup, Denmark). After incubation with biotinylated polyclonal rabbit anti-rat antibody (Dako, Glostrup, Denmark) and EnVision-HRP (Dako, Glostrup, Denmark) the signal was developed with AEC Substrate (Dako, Glostrup, Denmark) and samples were counterstained with Shandon Gill3 Hematoxylin (Thermo Scientific, Cheshire, UK). Staining for STAT3 phosphorylation was carried out using anti-mouse pSTAT3 antibody (Cell signaling, Danvers, MA) diluted 1:3,000 and the signal was amplified using the Tyramide Signal Amplification kit (Perkin Elmer, Boston, MA), developed with AEC substrate and counterstained with Shandon Gill3 Hematoxylin.

2.2.5.5 Quantification of Immunohistology

Magenta nuclear color reaction indicated STAT3 phosphorylation or positive stained neutrophilic cells in liver sections. Infiltrated neutrophils from liver sections of mice were quantified and positive cells were counted from 10 random high-powered fields with 20x magnification from 3-5 mice of each group. Cells stained positive for pSTAT3 in immunostained liver sections were also quantified by

counting 10 random high-powered fields of 20x magnification photomicrographs from 3-5 mice of each group.

2.2.6 Protein-biochemical methods

2.2.6.1 Cell lysis

50-100 mg of liver tissue originating from one murine liver lobe was given to 500 µl lysis buffer (500 mM NaCl, 50 mM Tris, pH7.4, 0.1 % SDS, 1 % NP-40) and was homogenized by 4-6 precellys ceramic balls using the precellys 24 shaker-system. Afterwards, reaction tubes containing the homogenized solution were constantly agitated for 1 h at 4°C. Subsequently, debris was removed by centrifugation (16,000 x g, 30 min, 4°C) and the supernatant was stored at -20°C.

2.2.6.2 Determination of protein concentration (BCA)

The protein concentration of cell lysates was determined using the BCA kit (Pierce). Solutions A and B were mixed (ratio 1:50) to give the BCA solution. Cell lysates were diluted 1:10 with PBS. 10 µl of the diluted cell lysate were mixed with 200 µl BCA solution in microtiter plates and incubated for 30 min at room temperature. A BSA standard curve was co-incubated ranging from 100 µg/µl to 2 µg/µl. The extinction of the samples was determined at 562 nm in a microtiter plate reader (Tecan, Maennedorf, Switzerland).

2.2.6.3 SDS-polyacrylamide gel electrophoresis

Separation of proteins was performed by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-Protean III system (BioRad). The size of the running and stacking gels was as follows:

Running gel:	height 4.5 cm, thickness 1 mm 10 % or 15 % acrylamide solution
Stacking gel:	height 1 cm, thickness 1 mm 5 % acrylamide solution 10-well combs

After complete polymerization of the gel, the chamber was assembled as described by the manufactures protocol. Up to 20 µl samples were loaded in the pockets and the gel was run at constant 120 V for 5 min and then at 180 V for the remainder. The gel run was stopped when the bromphenol blue line reached the bottom of the gel. Gels were subsequently subjected to Western blotting.

2.2.6.4 Western blot analysis

Proteins were transferred from the SDS-gel on a PVDF membrane (Amersham-Biosciences, Buckinghamshire, U.K.) using a MINI TRANSBLOT-apparatus (BioRad). The blotting sandwich was assembled as described in the manufactures protocol. Proteins were transferred electrophoretically at 4°C in blotting-buffer at 3-4 mA/cm^2 for 75 min.

2.2.6.5 Immunochemical detection of proteins on PVDF membranes

After electrophoretic transfer, the protein loaded membranes were removed from the sandwiches and placed with the protein-binding side up in plastic vessels. Membranes were immediately incubated with 25 ml blocking buffer for 1 h at RT. Afterwards, the primary antibody was added in the appropriate dilution overnight at 4°C. The primary antibody was removed by washing the membrane 3 x 10 min with TBS-T. The appropriate secondary antibody was applied for 1 h at RT and afterwards the membrane was washed again 3 x 10 min with TBS-T. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system (Amersham-Biosciences, Buckinghamshire, U.K.). Therefore the membrane was soaked for 3 min in detection solution and afterwards placed between two transparent plastic foils. The membrane was exposed and analyzed with the chemiluminescense camera system LAS-100 (Fujifilm, USA) for defined time periods.

2.2.7 Statistical analysis

Data are expressed as mean values ± SD; 3-10 mice were used per experimental group. Statistical analysis was performed by using a Student's unpaired *t* test (http://www.physics.csbsju.edu/stats/t-test.html). A p-value below 0.05 was considered statistically significant and denoted with one asterisk (*), whereas a

p-value below 0.01 was indicated with two asterisks (**) or (***) for a p-value below 0.001 (*p≤0.05; **p≤0.01 and ***p≤0.001), respectively.

3 Results

3.1 Impact of endogenous IL-6 in ConA-induced hepatitis

3.1.1 Highly dosed ConA application is accompanied by high mortality

Intravenous injection of 20 µg/g dosed ConA resulted in lower alanine transaminase (ALT) serum levels in IL-6 deficient mice compared to control mice 8 h after treatment (Fig. 5A). On the contrary, 24 h after application of 20 µg/g ConA IL-6 deficient mice exhibited increased ALT levels compared to control animals (Fig. 5B). Similar ALT levels in both groups of control mice and increased ALT levels from 8 to 24 h in IL-6 deficient mice indicated a time dependent development of liver damage and an important role of endogenous IL-6 during the onset of ConA-mediated hepatitis. Besides this observations, 30-40 percent of all used mice died within 8 or 24 h after application of 20 µg/g ConA (Tab.1). Therefore a lower dose of ConA (12.5 µg/g mouse) was used for all further experiments.

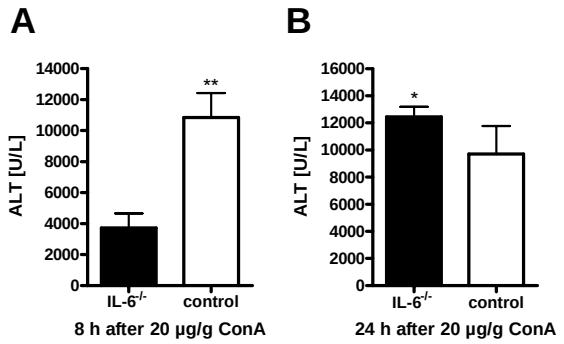

TABLE 1. Mortality rate after high dosage ConA (20 µg/g)

Experiment number	Genotype	Experiment duration	N beginning	N dead
1	IL-6$^{-/-}$	8 h	8	2
	wild-type		9	3
2	IL-6$^{-/-}$	24 h	6	3
	wild-type		7	4

Figure 5: High dosage ConA induced massive liver damage and lethality. Groups of male C57BL/6 mice and IL-6 deficient mice were challenged with a single injection of ConA (20 µg/g, i.v.) for (A) 8 hours and (B) 24 hours and ALT levels were determined from blood serum. The used number of animals and the number of dead animals during experimental duration is given in table 1 (Tab. 1). Up to 60 percent of all treated animals died within the experiment duration before samples could be taken. Values are shown as means ± SEM. *p≤0.05 and **p≤0.01.

3.1.2 Abrogation of classic IL-6 signaling but not IL-6 trans-signaling reduces ConA-mediated liver hepatitis

Intravenous administration of a single dose of ConA (12.5 µg/g mouse) resulted in lower alanine transaminase (ALT) serum levels in IL-6 deficient mice compared to control mice 8 h after treatment (Fig. 6A). However, 24 h and and 48 h after ConA application the amount of hepatocyte derived ALT in the serum of IL-6 deficient mice and control animals was comparable.

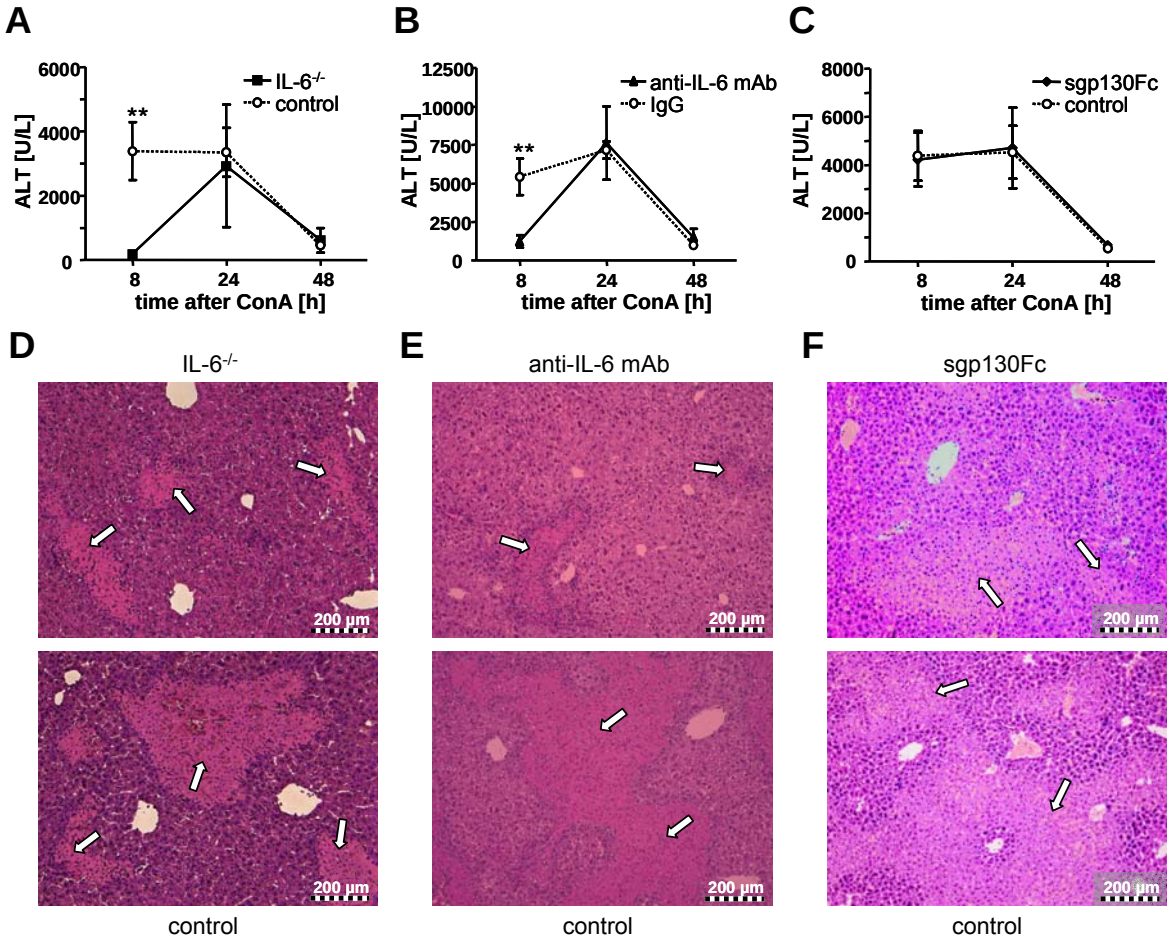

Figure 6: IL-6 signaling alters liver damage in ConA treated mice. Mice were intravenously injected with 12.5 µg/g ConA. ALT levels were determined form blood serum of (A) IL-6-deficient mice, (B) wild-type mice pretreated with an neutralizing anti-IL-6 antibody (200 µg/mouse, i.p.) 16 h before ConA injection and (C) wild-type mice pretreated with sgp130Fc (250 µg/mouse, i.p.) 16 h before ConA injection. C57BL/6 control animals remained untreated (A), were i.p. injected with control IgG (B) or PBS (C) 16 h before ConA treatment. Representative photomicrographs of H&E stained sections from (D) IL-6-deficient mice, (E) anti-IL-6 antibody pretreated mice and (F) sgp130Fc pretreated mice, 48 h after ConA injection. Massive necrosis is indicated by white arrows. Values are shown as mean ± SEM. **p≤0.01.

Nevertheless, histological examination of the liver by H&E staining displayed fewer and smaller areas of liver damage 48 h after ConA administration in IL-6 deficient mice compared to control mice (Fig. 6D). Generally, there was some variation in ALT levels, e.g. 24 h after ConA application (Fig. 6A). In addition, also ALT level variations between two sets of experimental data were noted (Fig. 6A&B). Still, liver damage in mice, which received a single injection of neutralizing anti-IL-6 mAbs 16 h before ConA was reminiscent of that found in IL-6 deficient mice (Fig. 6B&D). In contrast, liver damage in mice injected with sgp130Fc 16 h before ConA was not altered compared to control mice (Fig. 6C&F), indicating that initial ConA-mediated liver damage is at least partially mediated by IL-6 classic signaling but not by IL-6 trans-signaling.

Serum IL-6 levels were determined to survey an IL-6 induction during ConA-mediated liver damage. IL-6 levels were found to be strongly elevated and comparable in all groups of control mice 8 h after ConA application and decreased 24 h and 48 h after ConA challenge (Fig. 7A-C).

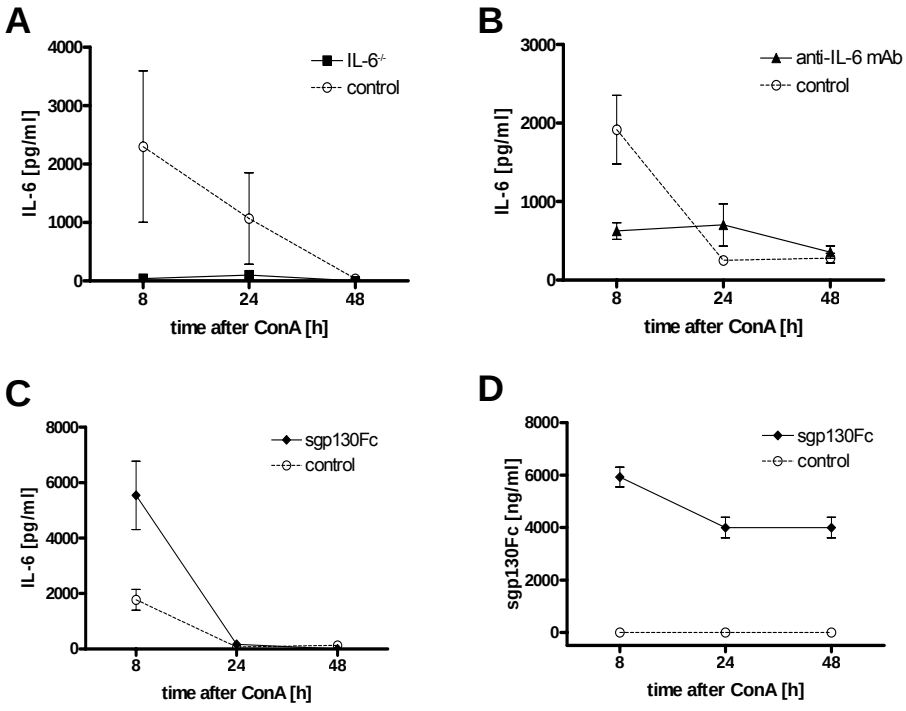

Figure 7: Serum IL-6 and sgp130Fc level after ConA application. IL-6 level from (A) IL-6-deficient mice, (B) anti-IL-6 antibody pretreated mice and (C) sgp130Fc pretreated mice were determined by ELISA after ConA challenge. (D) Serum sgp130Fc level in sgp130Fc pretreated mice after ConA injection.

In contrast, 3 different patterns of IL-6 levels were observed in IL-6 deficient-, anti-IL-6 mAb- and sgp130Fc-treated mice. As expected, no IL-6 was detectable in IL-6 deficient mice (Fig. 7A). Anti-IL-6 mAb treated mice exhibited slightly increased levels of IL-6 compared to control mice during all points of investigation (Fig. 7B), whereas sgp130Fc treated mice revealed a 3-fold increase of serum IL-6 8 h after ConA injection (Fig. 7C). Application of sgp130Fc was evidenced by measurement of serum sgp130 levels (Fig. 7D). However, in consideration of the observed liver damage severity, different IL-6 levels do not seem to be responsible since ALT level of IL-6 deficient and anti-IL-6 mAb treated mice were comparable and also IL-6 level in sgp130Fc treated mice and their corresponding control animals were similar.

3.1.3 Abrogated classic IL-6 signaling caused reduced STAT3 phosphorylation in the liver during ConA-mediated hepatitis

The expression of the IL-6R is limited to cell populations such as lymphocytes and hepatocytes whereas gp130 is ubiquitously expressed. Phosphorylation of STAT3 is one major downstream signaling event in IL-6 stimulated cells. Therefore, the phosphorylation status of STAT3 in hepatocytes was analyzed to elucidate whether IL-6-signaling in the liver is mediated by IL-6 classic or trans-signaling during ConA-induced processes. 8 h after ConA-challenge significantly reduced STAT3 phosphorylation was observed in IL-6 deficient mice and anti-IL-6 mAb treated mice but not in sgp130Fc treated mice compared to their respective control animals (Fig. 8A-C). Although numbers of pSTAT3 positive cells from immunostained liver sections (Fig. 8B) revealed variations between the control groups depending on the severity of induced liver damage (compare Fig. 6A&B), these results demonstrate that during ConA-mediated hepatitis the liver is a target of classic IL-6 signaling and not of IL-6 trans-signaling, although the liver clearly has the potential to conduct IL-6 trans-signaling (103,104).

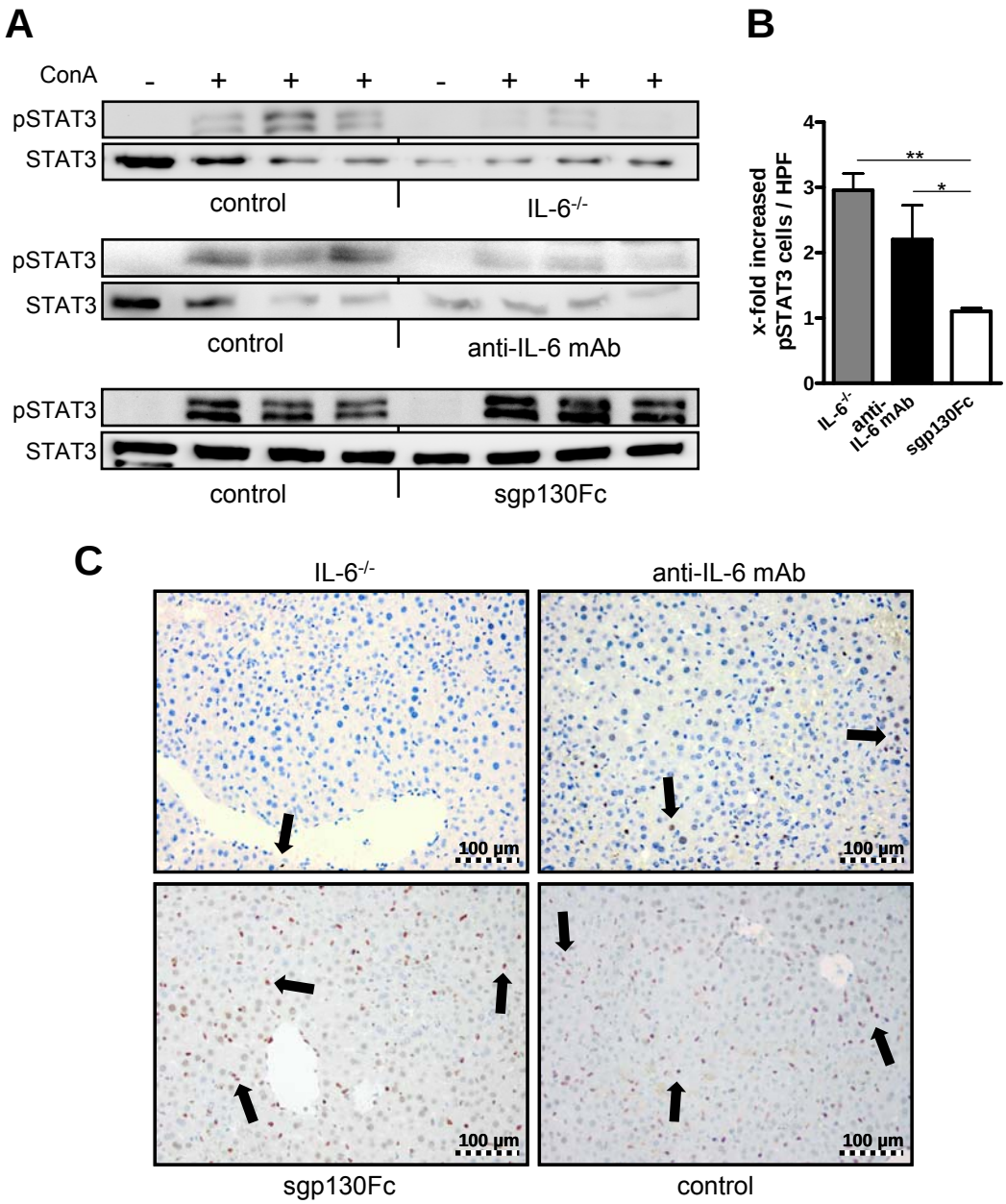

Figure 8: STAT3 phosphorylation in the liver 8 h after ConA. (A) Western blot analysis for pSTAT3 and STAT3 from liver lysates of mice, treated as described in Figure 4. Mice were sacrificed 8 h after ConA injection, liver extracts were prepared and Western blot analysis was performed. (B) Quantification of pSTAT3 positive cells, 8 h after ConA injection. Positive cells were counted from 10 random high-powered fields from 3 mice of each group and are shown as x-fold increase compared to their corresponding control mice. (C) Representative photomicrographs of liver sections from corresponding mice, stained for pSTAT3, 8 h after ConA treatment. Cells positively stained for pSTAT3 are exemplarily indicated by black arrows. Values are shown as means ± SEM. *p≤0.05 and **p≤0.01.

3.1.4 Reduced liver T-cell infiltration and activation in IL-6 deficient mice after ConA treatment

Activation of T-lymphocytes is a critical process in the pathophysiology of human autoimmune and viral hepatitis. $CD4^+$ T-cells and NKT cells, which have been shown to play an important role in ConA-induced hepatitis (92) were examined in IL-6 deficient mice and mice with blocked IL-6 trans-signaling due to injection of sgp130Fc before ConA treatment. Levels of $CD4^+$ cells from total liver lymphocytes were determined by FACS analysis of $CD3^+/CD4^+$ double positive cells. Levels of NKT cells from total liver lymphocytes were determined by gating $NK1.1^+/CD3^+$ double postive cells. The levels of activated $CD4^+$ T-cells from $CD3^+/CD4^+$ T-cells in the liver was examined by using the early activation marker $CD69^+$. Administration of ConA caused a significant increase of T-cell populations in all mice used (Fig. 9A-F).

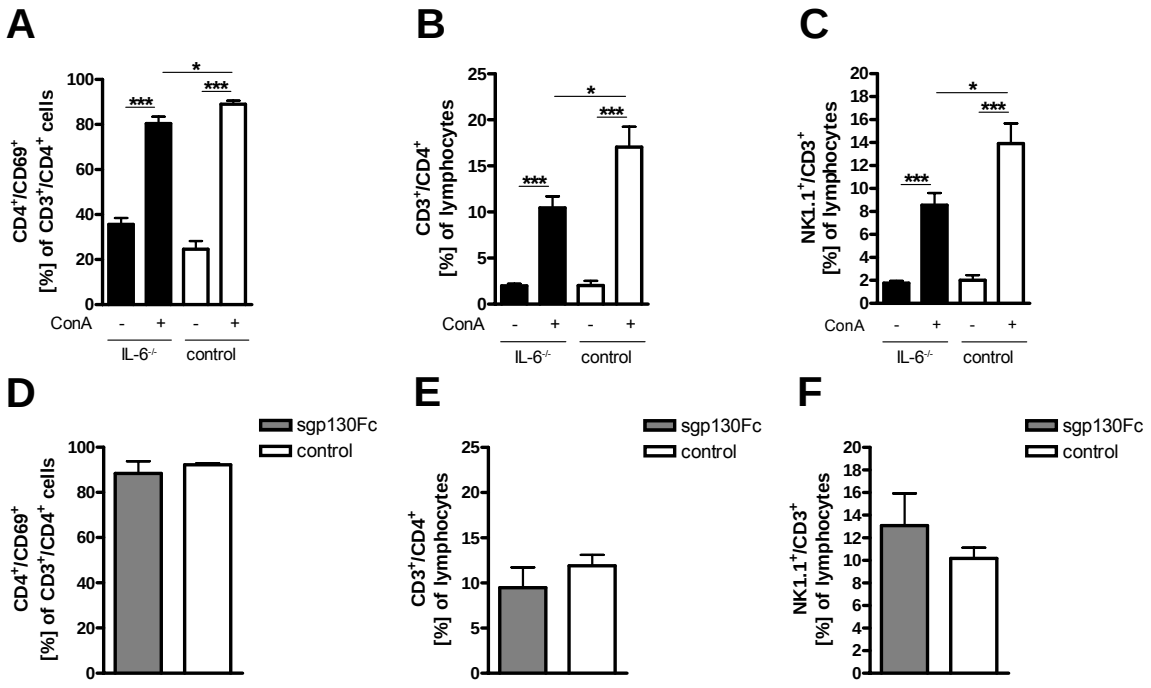

Figure 9: Relative T-cell populations in the liver 8 hours after ConA. Male C57BL/6 wild-type mice (n=5) and IL-6 deficient mice (n=5) were challenged with ConA (12.5 µg/g, i.v.) for 8 h. Control mice remained untreated. (A) Percentage of activated $CD4^+/CD69^+$ T-cells from $CD3^+/CD4^+$ T-cells. (B) Percentage of $CD3^+/CD4^+$ T-cells from total hepatic lymphocytes. (C) Levels of NKT cells from total liver lymphocytes. (D) Percentage of activated $CD4^+/CD69^+$ T-cells from $CD3^+/CD4^+$ T-cells. (E) Percentage of $CD3^+/CD4^+$ T-cells from total hepatic lymphocytes. (F) Levels of NKT cells from total liver lymphocytes in sgp130Fc treated mice after ConA. Values are given as means ± SEM. *p≤0.05 and ***p≤0.001.

However, percentages of $CD3^+/CD4^+$ T-cells and NKT cells from total liver-lymphocytes were reduced in IL-6 deficient mice compared to their wild-type control animals (Fig. 9B&C). In addition, levels of activated $CD4^+/CD69^+$ T-cells in the liver were reduced in IL-6 deficient mice (Fig. 9A), suggesting a role of IL-6 on activation of $CD4^+$ T-cells and the presence of NKT cells (Fig. 9A-C). Nevertheless, no differences in levels of activated $CD4^+$ T-cells, $CD3^+/CD4^+$ T-cells and NKT cells in sgp130Fc treated and control mice were found (Fig. 9D-F). These findings suggest that IL-6 dependent mechanisms for T-cell recruitment to the liver and activation of T-cells after ConA administration are mediated via IL-6 classic signaling and not trans-signaling.

3.1.5 IL-6 deficiency is accompanied by reduced numbers of liver neutrophils during ConA-mediated hepatitis

Neutrophil transmigration has been shown to be an IL-6 dependent process in many mouse models (105,106). In addition, neutrophils were shown to have an essential role in ConA-mediated hepatitis (87,107). Investigation of liver slides immunostained for neutrophils 8 h after ConA administration revealed reduced neutrophil numbers in IL-6 deficient mice and mice treated with anti-IL-6 mAb compared to sgp130Fc treated mice and control mice (Fig 10A). Quantification of positively immunostained neutrophils showed a 1.8 fold reduction of accumulated neutrophils in the liver of IL-6 deficient and anti-IL-6 mAb treated mice, whereas mice treated with sgp130Fc revealed no reduction of liver neutrophils compared to control mice (Fig. 10B). These data indicate that neutrophil recruitment after ConA challenge is mediated by classic IL-6 signaling but not by IL-6 trans-signaling.

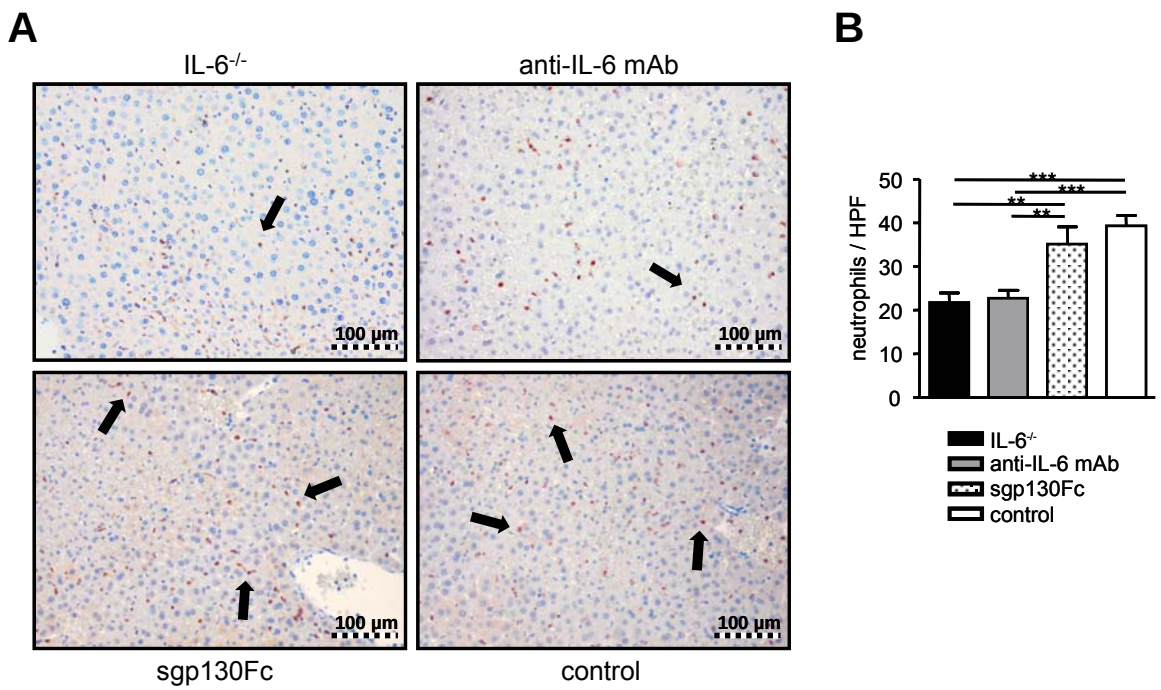

Figure 10: Neutrophil infiltration in the liver after ConA treatment. Groups of mice were treated as described in Figure 6. (A) Representative photomicrographs of liver slides from corresponding mice immunostained for neutrophils, 8 h after ConA treatment. Neutrophilic cells are exemplarily indicated by black arrows. (B) Quantification of infiltrated neutrophils from liver sections of corresponding mice, 8 h after ConA injection, counted from 10 randomly selected high-powered fields from 3 mice of each group. Values are shown as means ± SEM. **p≤0.01 and ***p≤0.001.

3.1.6 Neutrophil depletion completely protects from ConA induced liver injury

As previously reported (87), neutrophil depletion protected mice from ConA-mediated hepatitis. In fact, mice pretreated with neutrophil depleting Ly6G mAb showed normal serum ALT levels at all time points after ConA application (Fig. 11A). Furthermore, levels of two prominent members of chemoattractant cytokines, neutrophil-attracting chemokine KC and monocyte chemoattractant protein-1 (MCP-1) were examined. The levels of KC in neutrophil depleted mice were unaffected compared to control mice (Fig. 11C).

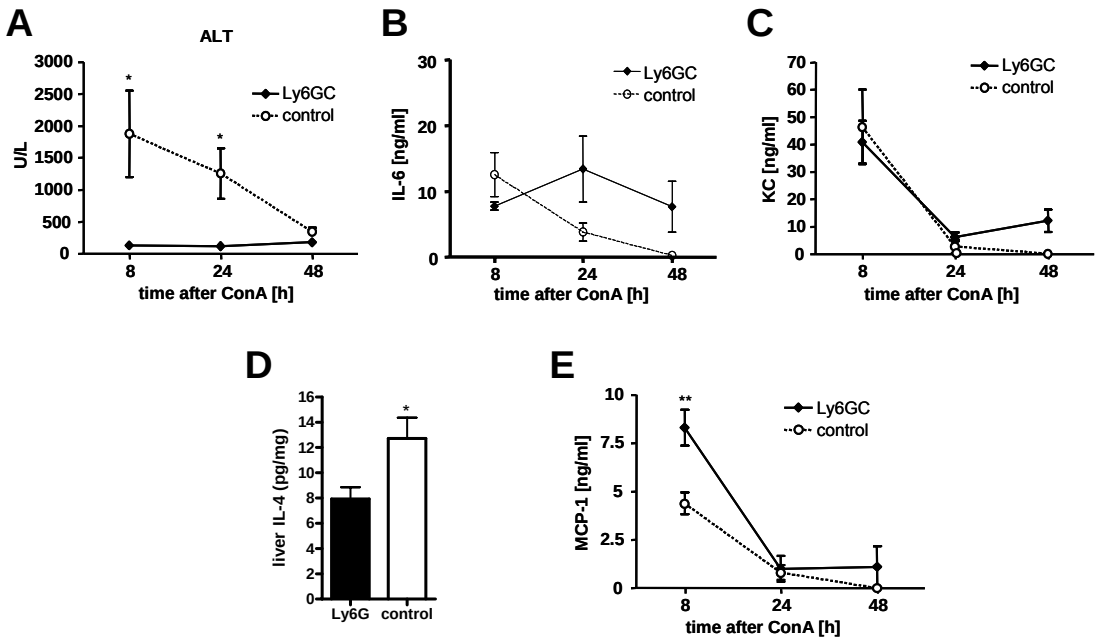

Figure 11: Neutrophil depletion protects from ConA induced liver damage. Groups of male C57BL/6 wild-type mice (n=5) were either injected (i.p.) with anti Ly6GC antibody (100 µg/mouse) or i.p. injected with 100 µl control PBS, 18 h prior to ConA i.v. injection (12.5 µg/g). (A) Blood samples were taken at the time points indicated and ALT levels were determined from blood serum. (B) Serum IL-6 levels, (C) serum KC levels and (E) serum MCP-1 levels were determined by ELISA. (D) Hepatic IL-4 levels were determined from liver lysates by ELISA, 8 h after ConA injection. Values are shown as means ± SEM. *p≤0.05 and **p≤0.01.

Accumulated neutrophils in the liver have been shown to inhibit the production of MCP-1 by Kupffer cells during *Listeria monocytogenes* infection (106) and MCP-1 has protective effects in ConA-mediated liver injury (95). Neutralization of MCP-1 led to an increase in liver damage and increased IL-4 secretion from activated liver-resident NKT cells, which exhibit proinflammatory effects in ConA-treated mice (95,108). Indeed, neutrophil depleted mice showed higher MCP-1 levels as compared to control mice during ConA-mediated hepatitis (Fig. 11E) and reduced IL-4 expression in the liver (Fig. 11D). Serum levels of IL-6 were not found to be significantly different in neutrophil depleted mice and control animals 8 h after ConA injection (Fig. 11B). These data suggest a direct connection between neutrophil infiltration, MCP-1 release and IL-4 expression during ConA-mediated hepatitis.

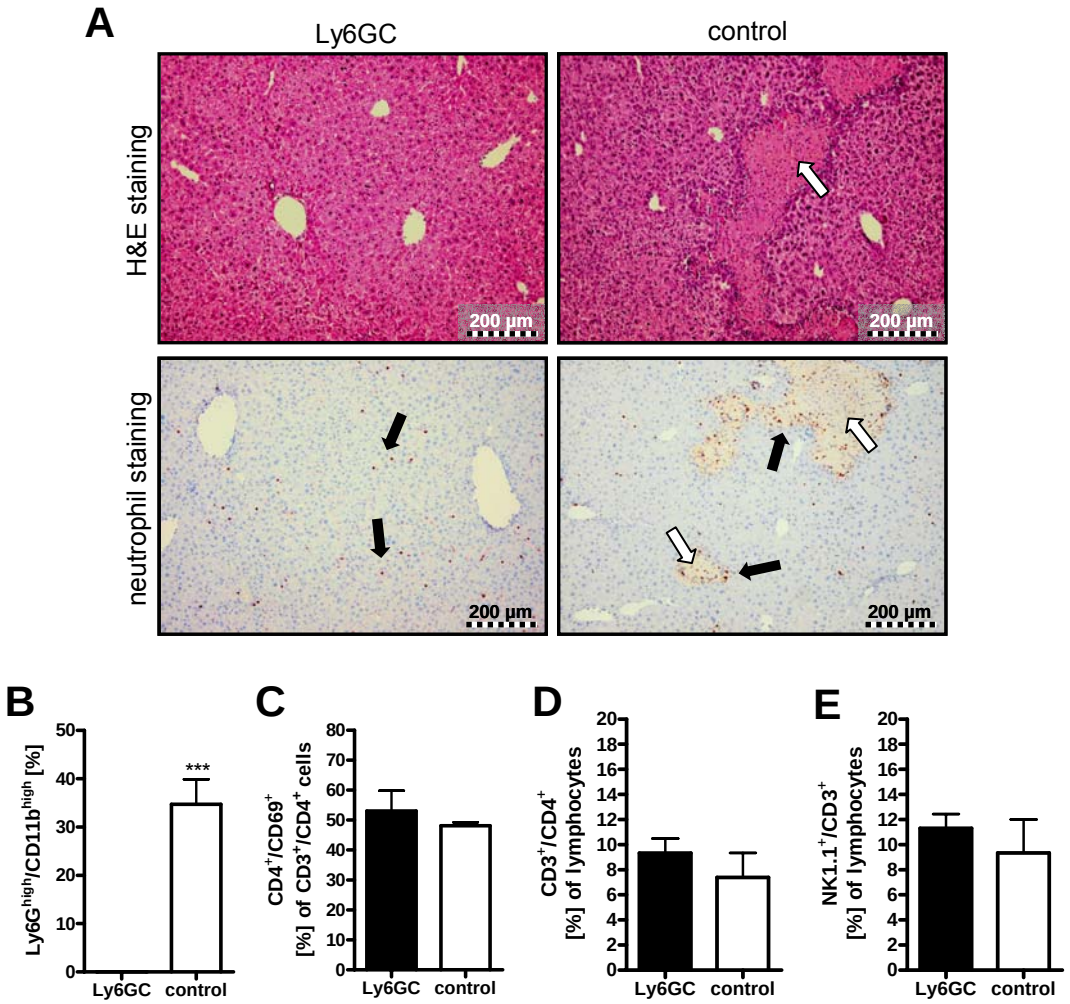

Figure 12: Distribution of lymphocytes in neutrophil depleted mice after ConA treatment. Mice were treated as described in Figure 8. (A) Representative photomicrographs of liver slides from corresponding mice H&E stained and immunostained for neutrophils, 48 h after ConA treatment. Neutrophilic cells are indicated by black arrows. Necrotic areas are indicated by white arrows. (B) Neutrophils in peripheral blood 8 h after ConA injection. (C) Percentage of activated $CD4^+/CD69^+$ T-cells from $CD3^+/CD4^+$ T-cells. (D) Percentage of $CD3^+/CD4^+$ T-cells from total hepatic lymphocytes. (E) Levels of NKT cells from total liver lymphocytes. Values are given as means ± SEM. ***p≤0.001.

Control analysis of neutrophil accumulation in the peripheral blood of depleted and ConA treated mice showed almost no neutrophils (Fig. 12B). In line, immunohistological investigation 48 h after ConA treatment showed no liver damage in neutrophil depleted mice but massive necrosis in control animals with neutrophils specifically localized at the border areas of liver damage (Fig. 12A). However, 48 h after ConA application also depleted mice displayed neutrophils inside the liver suggesting that reinfiltration takes place and neutrophils are only harmful during the first hours of ConA-induced hepatitis. Surprisingly, no

differences in hepatic $CD4^+$ T-cell and NKT cell levels were observed in neutrophil depleted mice compared to control animals (Fig. 12C-E), suggesting that the presence of activated neutrophils is not involved in recruitment and activation of T-lymphocytes after ConA challenge.

3.1.7 IL-6 deficiency results in increased hepatoprotective MCP-1 and decreased hepatodestructive IL-4 during ConA-mediated hepatitis

Since MCP-1 levels were increased and IL-4 level were reduced in neutrophil depleted mice during ConA-mediated hepatitis (Fig. 11D&E), corresponding cytokine levels were also analyzed in IL-6 deficient mice, anti-IL-6 mAb treated mice and sgp130Fc treated mice. 8 h after ConA-induced hepatitis MCP-1 levels were also significantly elevated in IL-6 deficient mice and mice treated with anti-IL-6 mAb but not with sgp130Fc (Fig. 13A). Even though abrogated classic IL-6 signaling led to diminished neutrophils in the liver, the neutrophil attracting chemokine KC was not altered (Fig. 13B). Reduction of liver-expressed IL-4 and TNF-α in IL-6 deficient and in anti-IL-6 mAb but not in sgp130Fc treated mice 8 h after ConA injection indicated that classic IL-6 signaling positively regulated IL-4 and TNF-α production (Fig. 13C-E). These findings suggested that reduced neutrophil numbers, increased MCP-1 level and reduced IL-4 level in the liver are crucial factors for the reduced ConA mediated liver damage in mice with blocked classic IL-6 signaling.

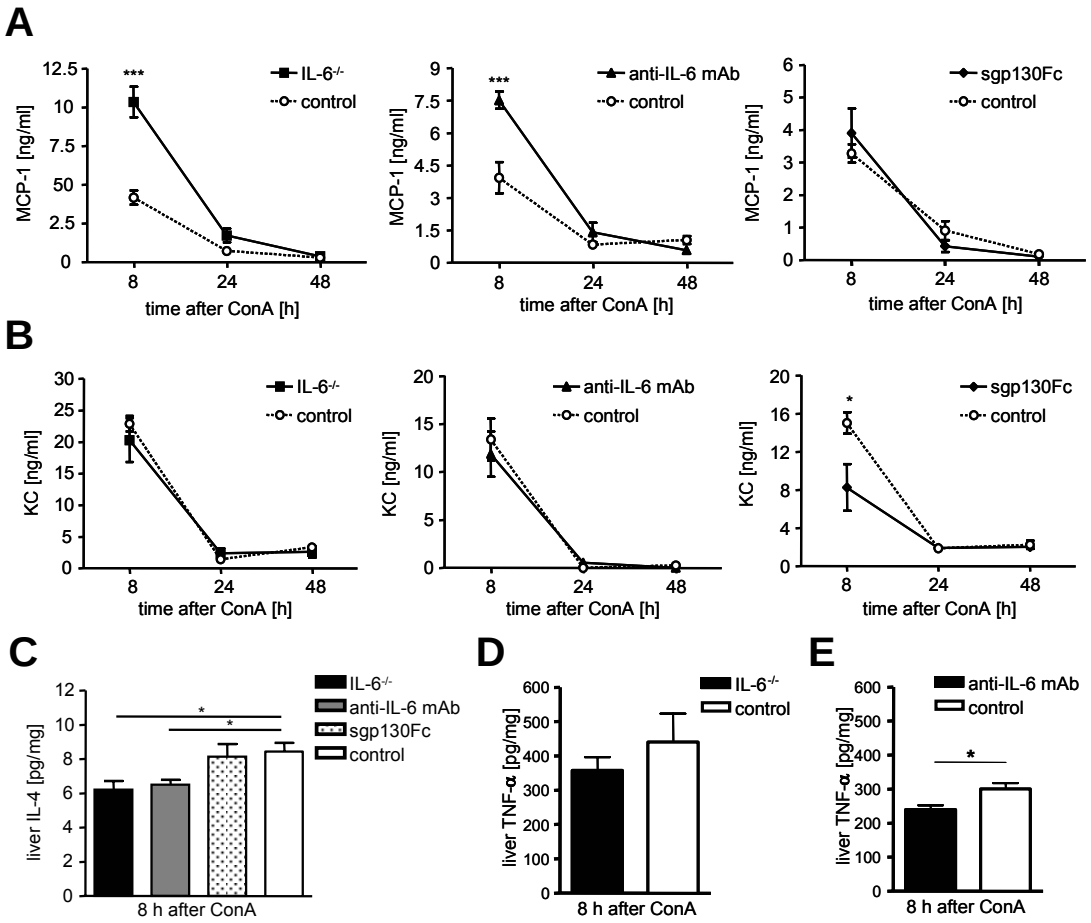

Figure 13: Inflammatory cytokine and chemokine levels in ConA challenged mice. Groups of mice were treated as described in Figure 6. (A) Serum MCP-1 levels and (B) serum KC levels were determined by ELISA. (C) Hepatic IL-4 levels and hepatic TNF-α levels from (D) IL-6 deficient and (E) anti-IL-6 antibody treated mice were determined from liver lysates by ELISA, 8 h after ConA injection. Values are shown as means ± SEM. *p≤0.05 and ***p≤0.001.

3.1.8 Deficiency of classic IL-6 signaling caused reduced levels of circulating neutrophils in the peripheral blood during ConA-mediated hepatitis

Neutrophil depleted mice displayed no elevated liver damage and no neutrophils in the peripheral blood after ConA treatment (Fig. 11A & Fig. 12B). In addition, reduced levels of circulating neutrophils in the peripheral blood were observed in IL-6 deficient mice during *Listeria monocytogenes* infection (109), indicating that endogenously produced IL-6 is required to mount an efficient neutrophilia. Therefore, also IL-6 deficient mice, anti-IL-6 mAb treated mice and sgp130Fc

treated mice were examined for neutrophil level in the peripheral blood 8 h after ConA application. An 5.4 fold increase of circulating neutrophils in the peripheral blood was observed in wild-type mice compared to untreated controls, which was reduced in IL-6 deficient mice (2.4 fold) and mice treated with anti-IL-6 mAb (3.2 fold) but not with sgp130Fc (5.8 fold) (Fig. 14A). These observations point out that classic but not IL-6 trans-signaling is responsible for neutrophilia during immune responses such as induced in ConA-mediated hepatitis.

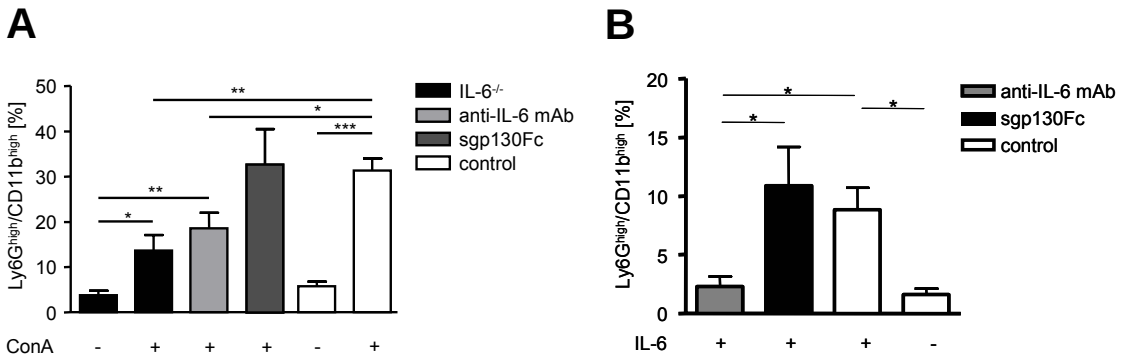

Figure 14: Neutrophil accumulation in the peripheral blood of ConA and IL-6 injected mice. Neutrophils in peripheral blood were determined by flow cytometry. (A) Neutrophils in peripheral blood determined 8 h after ConA injection. Mice were pretreated as described before and challenged with ConA (12.5 µg/g, i.v.) for 8 h. (B) Neutrophils in peripheral blood, 4 h after cytokine injection. 2 µg/mouse IL-6 with or without 200 µg/mouse anti-IL-6 mAb or 250 µg/mouse sgp130Fc were injected i.v. in mice (n=4). Values are shown as means ± SEM. *p≤0.05, **p≤0.01 and ***p≤0.001.

Intravenous injection of IL-6 has been described to induce neutrophilia with an early phase caused by mobilization of neutrophils into the peripheral blood from the marginated pool of the vascular endothelium and a late phase resulting from an accelerated bone marrow release of neutrophils (52,110). Thus, IL-6 with or without anti-IL-6 mAb or sgp130Fc was injected i.v. and levels of circulating neutrophils in the peripheral blood were analyzed after 4 h. Indeed, IL-6 treatment induced neutrophilia, which could be specifically inhibited with anti-IL-6 mAb but not with sgp130Fc (Fig. 14B), indicating that neutrophilia is mediated by classic IL-6 signaling but not by IL-6 trans-signaling. Taken together, these findings indicate that neutrophilia strongly contributes to the onset of liver damage and is primarily mediated by IL-6 during ConA induced hepatitis.

3.2 The dual mode of IL-6 action in ConA-induced hepatitis

3.2.1 IL-6 treatment before ConA challenge protects from liver damage

It has been shown that injections of recombinant IL-6, 24 - 3 h before ConA injection, completely prevented liver damage, whereas IL-6 administration 3 - 6 h after ConA challenge resulted in enhanced liver injury (90). In consequence, endogenous IL-6 present before ConA administration seems to be hepatoprotective, while IL-6 production after ConA injection promotes liver damage. Indeed, intravenous injection of recombinant IL-6, 3 h before ConA treatment, prevented liver damage and ALT serum increase compared to control mice (Fig. 15A). In addition also serum levels of MCP-1 (Fig.15B) and KC (Fig. 15C) were reduced in IL-6 treated mice. Since IL-6 serum levels were not altered in IL-6 protected mice after ConA challenge (Fig. 15D), these findings suggest a low potential of endogenous IL-6 for the induction of inflammatory processes in this mice. However, IL-6 has been shown to have an impact on neutrophil recruitment by soluble IL-6 receptor signaling during inflammation (97). In addition, neutrophil-depleted mice showed reduced $CD4^+$ T-cell recruitment to the liver and diminished liver injury after ConA administration (87). Suprisingly, no differences in levels of blood- and liver-accumulated neutrophils were observed (Fig. 15E&F), indicating that the hepatodestructive potential of neutrophils after injection of recombinant IL-6 was diminished. Some data indicate that the protective effect of IL-6 is due to STAT3 activation and subsequent suppression of IFN-γ signaling and induction of Bcl-X_L (92). In line, IL-6 has been shown to induce expression of the anti-apoptotic hepatic proteins FLIP and bcl-2 after Fas agonist treatment (111). Therefore, induction of bcl-2 and FLIP expression in the liver was examined in IL-6 treated mice and control mice 4 h after ConA injection. No noticeable differences were found in expression of bcl-2 and FLIP proteins (Fig. 15G), indicating that the protective effect of IL-6 treatment before ConA challenge might not be due to induction of anti-apoptotic events.

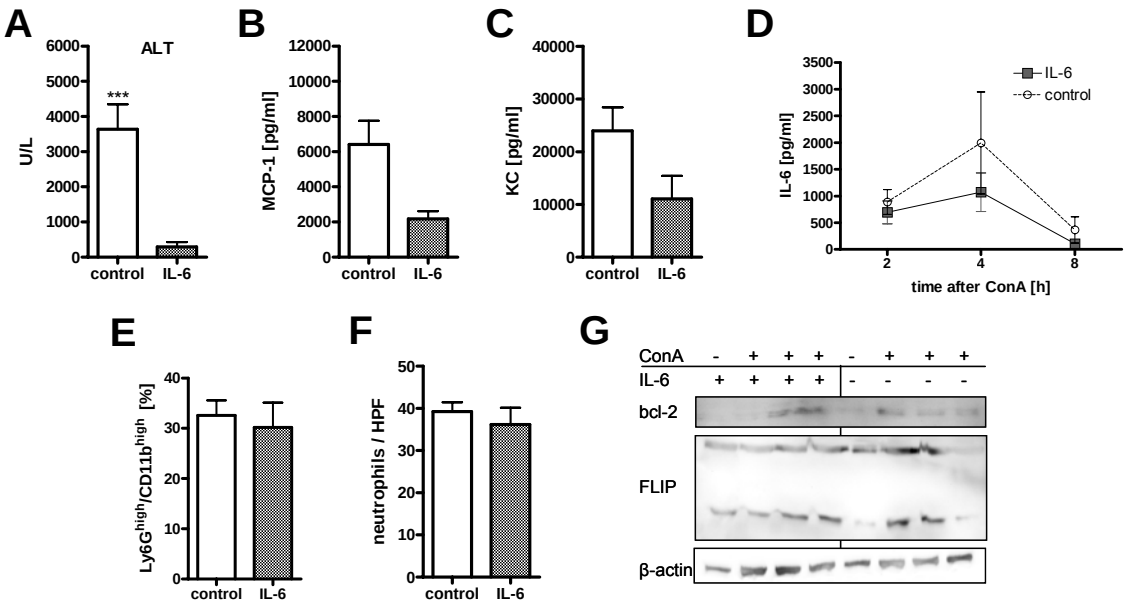

Figure 15: ConA-mediated hepatitis after IL-6 pretreatment. 3 h before ConA injection mice were intravenously injected with 2 µg recombinant IL-6. (A) Serum ALT levels, (B) serum MCP-1 levels, (C) serum KC levels and (D) serum IL-6 levels in IL-6 pretreated mice and control mice 8 h after ConA challenge. Levels of neutrophils in peripheral blood (E) and liver (F) 8 h after ConA injection. (G) Western blot analysis of anti-apoptotic bcl-2 and FLIP (2 splice variants shown) expression in the liver 4 h after ConA application.

3.2.2 IL-6 deficiency impairs ConA tolerance and results in reinfiltration of neutrophils

It has been shown that 8 days after a first ConA injection of ConA, mice become tolerant against ConA restimulation (112). Since IL-6 deficient mice showed reduced ConA-mediated liver damage, also tolerance induction towards ConA was investigated in IL-6 deficient mice. 8 days after the first ConA challenge, mice were rechallenged with ConA. IL-6 deficient and control mice showed no increase of ALT level 8 h after the second ConA challenge. However, 24 h after the second ConA-challenge IL-6 deficient mice showed increased blood ALT level (Fig. 16A) and increased liver damage as shown by immunohistochemical analysis, whereas control mice remained unaffected (Fig. 16C). suggesting that IL-6 plays a major role in ConA tolerance development. Evidence for the importance of IL-6 signaling during ConA tolerance is also given by IL-6 levels in the serum of re-treated mice. An abolished re-release of endogenous IL-6 to the serum in protected mice 8 and

24 h after ConA re-treatment reflects the dual mode of IL-6 action with initial harmful and long-term protective effects (Fig.16B).

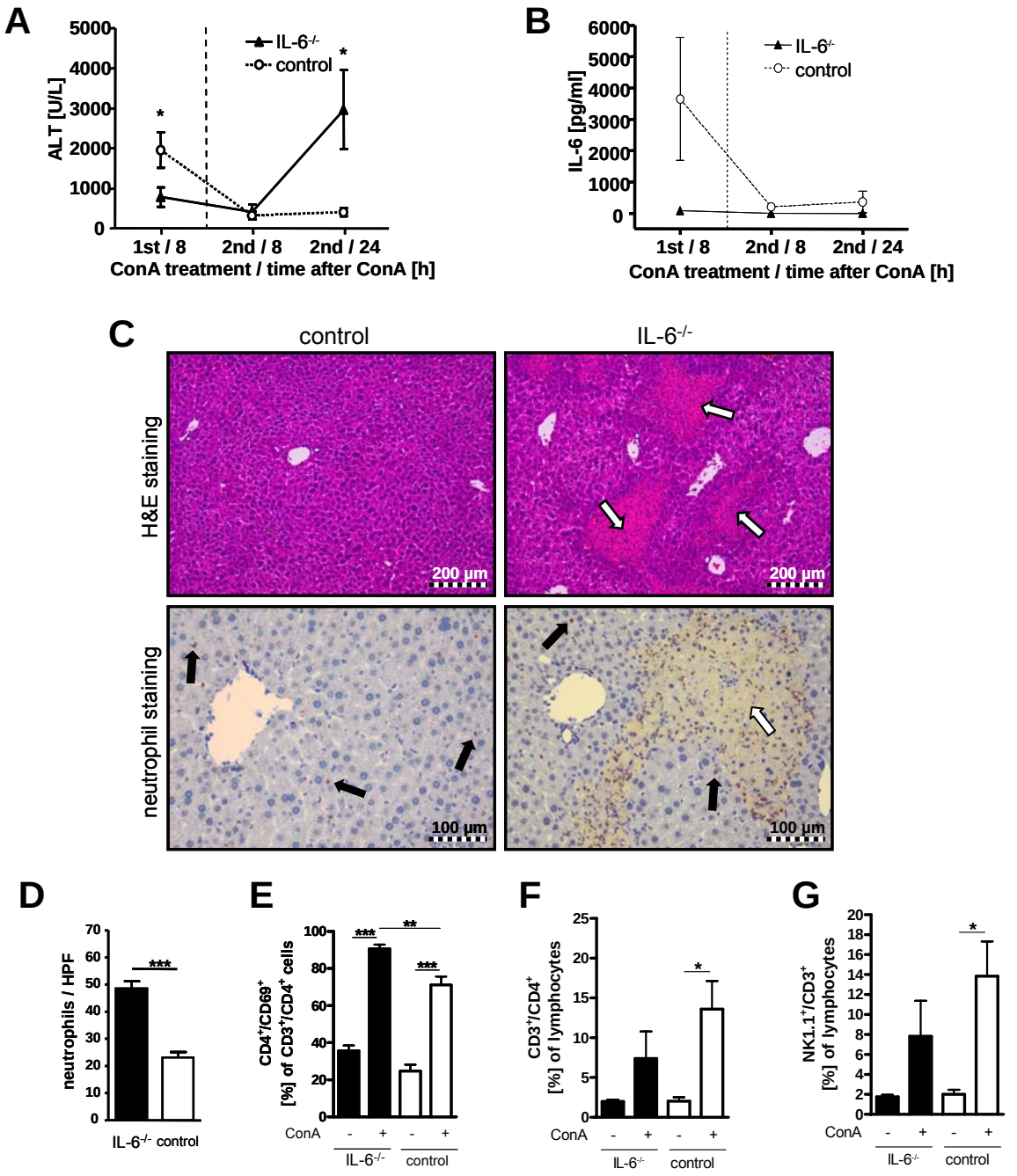

Figure 16: Liver damage and immune cell populations after ConA rechallenge. (A) Nine days after ConA treatment (12.5 µg/g, i.v.) wild type (n=5) and IL-6 deficient (n=5) were challenged with a second injection of ConA (12.5 µg/g, i.v.) for 24 h and ALT levels were determined. (B) Serum IL-6 level determined by ELISA. (C) H&E stained and neutrophil immunostained sections from corresponding mice 24 h after second ConA injection. Necrotic areas are indicated by white arrows. Neutrophilic cells are indicated by black arrows. (D) Infiltrated neutrophils were quantified by counting 10 random high-powered fields from 3 mice of each group. Levels of (E) activated $CD4^+$ T-cells, (F) $CD3^+/CD4^+$ T-cells and (G) NKT cells estimated by flow cytometry analysis. Values are shown as means ± SEM. *p≤0.05; **p≤0.01 and ***p≤0.001.

Surprisingly, 24 h after rechallenge with ConA, tolerant mice showed 1.7 times less neutrophils in the liver compared to the first ConA-challenge, whereas IL-6 deficient mice had 2.2 times higher numbers of liver neutrophils than control mice (Fig. 16C&D). In line to the first challenge with ConA, percentages of $CD3^+/CD4^+$ T-cells and NKT cells from total liver-lymphocytes were reduced in control mice and in IL-6 deficient mice (Fig. 16F&G) but levels of activated $CD4^+/CD69^+$ from $CD3^+/CD4^+$ T-cells were increased in IL-6 deficient mice (Fig. 16E). These findings emphasize the dual role of IL-6 in ConA-mediated processes and also suggest mechanisms other than IL-6 signaling, for neutrophil and T-cell activation in ConA tolerance.

4 Discussion

4.1 Endogenous IL-6 contributes to liver damage during the onset of ConA-mediated hepatitis

IL-6 is a multifunctional cytokine involved in pro- and anti-inflammatory processes during disease progression. Intravenous injection of ConA into mice induced massive tissue damage limited to the liver and led to an increased release of IL-6. IL-6 was shown to play a dual role in ConA-induced hepatitis. Exogenous IL-6 has been reported to protect against the disease, when IL-6 was injected 24 to 3 h before ConA-induced liver injury, but to amplify disease manifestations when IL-6 was administered 3 to 6 h after ConA injection (90,113,114). In hepatocytes, exogenous IL-6 induced the production of acute-phase proteins, and serum amyloid A (SAA) has been found to be protective for hepatocytes in ConA-mediated hepatitis (115).

The role of endogenous IL-6 during ConA-mediated hepatits has been examined by two different approaches in IL-6 deficient mice and mice treated with anti-IL-6 antibodies at various time points before and after ConA treatment (92,96). These reports claim that IL-6 deficient mice were more susceptible to liver injury after high-dose application of ConA (92). From antibody studies it was concluded that endogenous IL-6 present before ConA challenge as well as that induced in the very early phase after ConA injection was hepatoprotective. IL-6 produced beyond the early phase was hypothesized to be harmful to hepatocytes (96). In both studies a 20 µg/g ConA dosage was used to examine the role of IL-6. 24 h after ConA injection IL-6 deficient mice were found to have increased ALT levels compared to wild-type mice. Indeed, own results 24 h after high dosed ConA application mice showed increased ALT levels compared to control animals (Fig. 5B). However, a closer inspection of liver damage 8 h after high dosed ConA application revealed significantly reduced ALT levels in IL-6 deficient mice compared to wild-type mice (Fig. 5A). Furthermore, application of 20 µg/g ConA induced massive lethality in mice (Fig. 5, Tab.1). Since liver diseases, mimicked by ConA application like autoimmune hepatitis and viral hepatitis last from days to months before they become life-threatening, a dosage of 20 µg/g seemed to be

inadequate for investigation. In addition, ConA administration has been shown to induce prominent intrasinusoidal hemostasis with massive erythrocyte agglutination (116), which might be overwhelming in high dosed ConA approaches and might also induce lethality independent of toxic liver effects. Therefore, a lower dose of ConA with 12.5 µg/g was used in all further examinations in order to be as close as possible at conditions of immune-mediated liver diseases. Nevertheless, findings indicate that hepatic damage by ConA and the role of IL-6 within this model was dose and time dependent.

Administration of a lower dose of ConA (12.5 µg/g) also led to significant but not lethal hepatitis, as demonstrated by a profound occurrence of serum ALT 8 h and 24 h post-ConA injection compared to untreated mice (Fig. 6), which at 48 h almost returned to base-line levels. However, an unexpected distinctive reduction in serum ALT levels 8 h after ConA administration in IL-6 deficient mice was observed (Fig. 6A). Histological examination of the liver by H&E staining of fixed liver sections showed large areas of necrotic liver damage 48 h after ConA administration in wild-type mice, whereas IL-6 deficient mice showed less and smaller areas of damage (Fig. 6D). To verify the data found in experiments with IL-6 deficient mice, a single injection of neutralizing anti-IL-6 antibody was applied to wild-type mice 16 h before ConA administration. Inhibition of IL-6 signaling resulted in reduced liver damage as shown by reduced ALT levels compared to control animals (Fig. 6B). Histological examination of the liver displayed markedly less and smaller areas of liver damage 48 h after Con-A administration (Fig. 6E). Taken together, these findings indicate that independent of ConA-dosage endogenous IL-6 contributes to liver damage during the first hours of ConA-mediated hepatitis progression and also influences the degree of severity. To further elucidate how IL-6 signaling contributes to the onset of ConA-mediated hepatits downstream activation of STAT3, induction of immune-response related cytokines and activation of immune cells was examined.

IL-6 activation of STAT3 in hepatocytes plays an important role in protection against liver injury induced by various toxins (58,92,111). Western blot analysis and histochemical analysis demonstrate that attenuated STAT3 signaling in IL-6 deficient and anti-IL-6 anitbody treated mice (Fig. 8) correlated with liver damage (Fig. 6), suggesting that reduced IL-6/STAT3 signaling protected against liver

damage in ConA-mediated hepatitis. IL-6 is the major cytokine responsible for STAT3 activation in liver regeneration (58) and CCl4-induced liver injury (117), since such activation is not detecable in IL-6 deficient mice under these conditions. However, STAT3 activation was reduced but not abolished in IL-6 deficient and anti-IL-6 antibody treated mice (Fig. 8), suggesting that other cytokines than IL-6 are also involved in STAT3 activation during ConA-induced hepatitis. Indeed, IFN-γ may partially contribute to STAT3 activation in this model, since injection of IFN-γ has been shown to induced significant activation of STAT3 in the liver of mice (92).

The molecular and cellular mechanisms underlying ConA-induced hepatitis have been extensively investigated, but are still not fully understood. No cellular receptor for ConA molecules has been defined yet and mutiple cells and cytokines have been implicated in ConA-mediated hepatitis, suggesting that T-cell mediated hepatitis results from complex interactions of multiple cell types and cytokines. Although $CD4^+$ T-cells are key players in this model, several cell depletion studies showed that other cell types including NKT cells, Kupffer cells and neutrophils are also required to induce an intense ConA-mediated hepatitis (39). The major role of $CD4^+$ effector T-cells has been well described, e.g. mice pretreated with a T-cell specific immunosuppressive drug failed to develop massive cellular recruitment and hepatic inflammation in response to ConA (85). In addition, studies with heparin treatment before ConA challenge indicated that it was neither the agglutination capacity nor the sugar specificity per se that determined the in vivo hepatoxic potential of the lectin ConA (116). It appeared that ConA substituted for the signal provided by macrophages through peptides bound to MHC class II molecules. Indeed, it was shown that ConA bound to the MHC of target cells (118). The involvement of macrophages in ConA hepatitis was not only shown by the absence of ConA-inducible lesions in macrophage-depleted animals but also by the morphological demonstration of macrophage-lymphocyte interactions. Thus, this experimental disease is probably produced by both lymphocytes and macrophages. The protection by anti-CD4 antibodies against ConA suggested a mechanism in which $CD4^+$-bearing $T_{Helper}(T_H)$-cells recognized the ConA-modified MHC structures of macrophages and became activated. Such a mechanism, i.e. T_H-cell activation followed by inflammatory tissue destruction, was also told to be responsible for the autoimmune disease rheumatoid arthritis (119). The finding

that the liver is the target organ of ConA-induced toxicity *in vivo* may be due to the fact that this organ contains the majority of macrophages in the body, named Kupffer cells. After intravenous injection of ConA, this resident macrophage population exposed to the circulation is primarily reached by the lectin. Therefore, it also seems likely that T-cell activation first starts within the liver and thus explains the distinct organotropy of ConA in terms of toxicokinetics.

Besides NKT cells and macrophages the liver contains a large resident and migratory population of leukocytes that provide immunosurveillance against foreign antigens. This hepatic population can be rapidly expanded in response to infection or injury by recruiting leukocytes from circulation. Moreover, acute and chronic hepatitis is pathologically characterized by a prominent infiltration of lymphocytes to the liver (120). Neutrophils, the numbers of which increase significantly after ConA administration, are also involved in the initiation and propagation of ConA-induced hepatitis. It has been demonstrated that neutrophil depletion reduced $CD4^+$ T-lymphocyte infiltration and completely prevented the development of hepatitis, suggesting a major role for neutrophils in the pathogenesis of ConA-mediated hepatitis (87,107). Besides their role in lymphocyte attraction, neutrophils themselves can act as effector cells and cause apoptosis and necrotic tissue destruction by massive secretion of proteases or reactive oxygen species (121-123). Indeed, pathology analysis by immunohistochemistry in the livers of ConA-treated mice showed neutrophil infiltration in pericentral and periportal areas with spotty, focal and submassive necrosis (Fig. 10A & 12A). Quantification of these neutrophils revealed significantly reduced numbers of infiltrated neutrophils in the livers of IL-6 deficient mice and mice treated with anti-IL-6 antibody before ConA challenge (Fig. 10B), pointing to a correlation of abrogated IL-6 signaling, reduced neutrophil numbers in the liver and the reduced liver damage marker ALT. Although neutrophil numbers in the liver were reduced in ConA-treated IL-6 deficient mice with abrogated classic IL-6 signaling, unchanged levels of the neutrophil attracting chemokine KC were observed (Fig. 13B), which indicated that neutrophil recruitment processes into the liver were not affected by IL-6 blockade. This hypothesis was underlined by investigation of neutrophil levels in the peripheral blood of mice 8 h after ConA injection. In the circulation, mice with abrogated IL-6 signaling again revealed reduced neutrophil numbers compared to control mice (Fig. 14A) indicating that

IL-6 signaling rather contributed to neutrophil release to the circulating system than neutrophil recruitment to organs. In previous studies it has been shown that IL-6 induced a release of neutrophils from the bone marrow (124). Indeed, intravenous injection of recombinant IL-6 to mice resulted in increased neutrophilic levels in systemic circulation (Fig. 14B). The main source of IL-6 is thought to be Kupffer and hepatic sinusoidal endothelial cells but also liver-associated T-cells have been reported to secrete IL-6 upon ConA application (13,125,126). Analysis of IL-6 signaling during ConA-mediated hepatitis revealed that IL-6 signaling was responsible to mount an efficient neutrophilia which than contributed to destructive ConA-effects and presents a mechanism of IL-6 during ConA-mediated hepatitis that is not restricted to the liver.

Furthermore, levels of NKT cells, $CD4^+$ T-cells and activated $CD4^+$ T-cells, which were all described to be harmful during ConA-mediated hepatitis were found to be diminished in IL-6 deficient mice compared to control animals (Fig. 9A-C). In addition, neutrophil depleted mice, which were found to be completely protected from ConA-induced liver damage, exhibited no alterations in levels of NKT and $CD4^+$ T-cells compared to control animals (Fig. 12). These two findings indicate that IL-6 is involved in and necessary for T-cell recruitment to the liver but activation of these cells' harmful potential might be counteracted by interaction with neutrophils and therefore presents another, indirect way of IL-6 signaling during ConA-mediated hepatitis.

In addition to cellular responses also immunoregulatory cytokines play a central role in the pathogenesis of ConA-mediated hepatitis with studies pointing to a proinflammatory role of TNF-α, IFN-γ, IL-12 and IL-4, and a protective role of IL-10 and MCP-1 (127). MCP-1 levels were found to be reduced in IL-6 deficient mice and mice treated with anti-IL-6 antibody 8 h after ConA treatment compared to control mice (Fig. 13A). Also neutrophil depleted mice showed reduced serum MCP-1 levels compared to control animals 8 h after ConA application. In a *Listeria monocytogenes* infection model, phagocytosis of neutrophils by Kupffer cells has been claimed to inhibit MCP-1 expression by Kupffer cells (106). A protective effect of MCP-1 in ConA-mediated hepatitis was demonstrated since neutralization of MCP-1 led to an increase in liver damage and increased hepatodestructive IL-4 secretion from activated liver-resident NKT cells (95). Recombinant MCP-1 was

able to reduce IL-4 production *in vitro* by ConA-costimulated NKT cells (95). IL-4 expression was also found to be reduced in IL-6 deficient and anti-IL6 antibody treated mice (Fig. 13C). Therefore, IL-6 may again contribute to hepatocyte damage by indirect neutrophil-mediated processes affecting macrophage expression of hepatoprotective MCP-1 and subsequently altered IL-4 expression in NKT cells.

4.2 IL-6 trans-signaling has no impact during hepatodestructive events in ConA-induced hepatitis

Application of sgp130Fc has been shown to have beneficial effects in animal models of inflammation (65), autoimmunity (81) and cancer (82) pointing to the importance and abundance of IL-6 trans-signaling in biological processes. In an air-pouch model of acute inflammation, transgenic sgp130Fc overexpressing mice have been shown to exhibit impaired leukocyte recruitment and reduced levels of MCP-1. Infiltrating neutrophils were the main source of soluble IL-6 receptor (128,129). Double transgenic mice for IL-6 and the soluble IL-6R had a higher liver weight and showed extramedullary hematopoiesis in liver and spleen and developed multifocal hepatocellular hyperplasia (74,103). IL-6 levels have been shown to be strongly increased during chemical liver damage induced by substances such as D-Galactosamine (130). Blockade of IL-6 trans-signaling by sgp130Fc showed that liver regeneration and regulation of glycogen consumption in the D-Gal liver toxicity model is mediated by IL-6 and the soluble IL-6R. These data showed that IL-6 trans-signaling processes in the liver are possible despite expression of membrane-bound IL-6R on liver cells including hepatocytes (104).

Taken together, these findings led to the assumption that IL-6 trans-signaling processes may also be of particular importance during ConA-mediated hepatits since the role of classic IL-6 signaling was discussed in the previous chapter. Therefore, the role of IL-6 trans-signaling was investigated during ConA-mediated hepatitis using IL-6 trans-signaling inhibitor sgp130Fc. Interestingly the protection observed in IL-6 deficient and anti-IL-6 antibody treated mice was not mediated by IL-6 trans-signaling, since mice pretreated with sgp130Fc before ConA challenge

showed liver damage comparable to control mice (Fig. 6C&F). Even though liver cells including hepatocytes and Kupffer cells express IL-6R and are therefore a likely target of classic IL-6 signaling, the liver is also a target of IL-6 trans-signaling. In this setting an activation of IL-6 trans-signaling enhanced stimulatory effects of IL-6 (131). Since hepatocytes express far more gp130 than IL-6R on the cell surface, the total number of activated gp130 molecules is higher when IL-6 plus sIL-6R is present (132). This effect might also been found during ConA hepatitis, since sgp130Fc pretreated mice displayed slightly but not significantly reduced STAT3 phosphorylation compared to control animals (Fig. 8). However, no differences in neutrophil recruitment to the liver and neutrophil release to circulation was observed in sgp130Fc mice compared to control mice (Fig. 10 & 14). Also, levels of NKT cells and $CD4^+$ T-cells were not altered in IL-6 trans-signaling blocked animals compared to controls (Fig. 9D-F). These findings indicated that IL-6 trans-signaling has only minor influence during the first onset of immune-mediated processes in the ConA-mediated hepatitis model. However, one result was found to be different in sgp130Fc treated mice compared to control animals and IL-6 deficient mice. Serum KC levels were significantly diminished in sgp130Fc mice (Fig. 13B), suggesting that IL-6 trans-signaling enhances induction of neutrophil attractant proteins by liver cells after ConA-injection. Nevertheless, this effect seemed to be without effect in the complex immunological events induced by ConA since diminished KC levels had no influence on neutrophil infiltration (Fig. 10).

4.3 Dual role of IL-6

The role of IL-6 in Con-A induced liver injury is confusing with hepatoprotective effects when IL-6 is injected before Con-A induced liver injury, but hepatodestructive when IL-6 is administered after Con-A injection (90) (114) (133). A similar protective effect of pre- but not post-treatment with IL-6 has also been reported in mice given a lethal challenge with endotoxin (134). Mice pretreated by intravenous injection of recombinant IL-6 were completely protected from ConA-induced liver damage (Fig. 15A). IL-6 serum levels were found to be elevated in IL-6 pretreated and control mice (Fig. 15D) indicating that IL-6 pretreatment had

no effect on IL-6 release after ConA injection. Levels of liver infiltrating neutrophils and circulating neutrophils were also equally elevated in IL-6 protected mice and control animals with severe liver damage (Fig. 15E&F). However, a second line of neutrophils, released from the bone marrow after first release stimulus by recombinant IL-6, has been shown to exhibit altered properties with high levels of F-actin and less deformability (124). In addition, MCP-1 and KC serum levels in IL-6 protected mice after ConA challenge were reduced compared to not protected mice (Fig. 15B&C). Therefore, the following mechanism is likely. IL-6 pretreatment induced release of neutrophils from the bone marrow. These neutrophils infiltrated the liver without doing any harm to hepatocytes since no antigenic ConA was attached to liver cell surfaces. After ConA was injected, a second wave of neutrophils was released to the circulation with reduced potential to induce liver damage due to altered harmful properties. Primary neutrophils were already taken up and digested from macrophages, indicated by reduced MCP-1 levels in IL-6 pretreated mice. Consequently, no harm was done to hepatocytes by getting in contact with ConA. A protective effect of IL-6 treatment before ConA challenge might thus be due to pre-release and clearance of neutrophils with effector potential or high protease/reactive oxygen species load and release of immature neutrophils without harmful properties subsequent to ConA challenge.

Induction of anti-apoptotic protein expression in hepatocytes was presumed to be another mechanism contributing to IL-6/STAT3-mediated protection against ConA-induced hepatitis, since it has been implicated in protection against hepatocyte death induced by a variety of hepatoxins, including CCl4 (117), Fas ligand (111) and alcohol (92). However, the protective effects of IL-6/STAT3 were unlikely to be mediated by induction of anti-apoptotic bcl-2 and FLIP protein expression in hepatocytes because similar activation was observed in mice pretreated with recombinant IL-6 and in control mice (Fig. 15G) suggesting that protection by IL-6 in ConA-mediated hepatitis is rather due to reduced induction of proapoptic cytokines like TNF-α than activation of anti-apoptotic genes.

4.4 Essential role of IL-6 signaling in tolerance development towards ConA-induced hepatitis

The liver is a major presentation site of antigens causing T-cell inactivation, tolerance induction and apoptosis, likely due to the need of maintaining immunological tolerance towards gut-derived food antigens (135). The overall predisposition of intrahepatic T-cell responses toward tolerance might account for survival of liver allografts without immuno-supression and persistance of liver pathogens, i.e. hepatitis B or C virus (136). In previous studies, the tolerogenic mechanisms of the liver have been examined predominantly with a focus on T-cells (137). A single injection of a sublethal dose of ConA to mice induced tolerance toward ConA-induced liver damage within 8 days pointing to suppression of T_H1 responses and increased IL-10 production being crucial for the tolerogenic state (112). Furthermore, IL-6 levels have been shown to be at baseline levels after a retreatment with ConA, suggesting that IL-6 had no influence in tolerance-mediating processes. Indeed, IL-6 levels were found not elevated after a second challenge with ConA (Fig. 16B). In addition, IL-6 deficient mice and control mice revealed baseline ALT levels 8 h after ConA rechallenge. Suprisingly, 24 h after ConA rechallenge, IL-6 deficient mice were found to develop severe liver damage defined by increased ALT levels and massive necrosis in H&E stained liver sections (Fig. 16A&C). Besides, severe liver necrotic areas were found accompanied by strong accumulation of neutrophils (Fig. 16C&D), suggesting that this time IL-6 independent mechanisms are responsible for efficient neutrophil release from the bone marrow. Furthermore, levels of $CD4^+$ T-cells and NKT cells were found slightly but not significantly reduced in affected IL-6 deficient mice compared to control mice (Fig. 16 F&G), pointing to a minor role of IL-6 signaling in T-cell recruitment processes during ConA rechallenge. In contrast, activation of $CD4^+$ T-cells was markedly increased in IL-6 deficient mice compared to control animals (Fig. 16E) pointing to a key function of IL-6 mediated T-cell activation during ConA induced hepatitis. Coherently, it has been demonstrated that co-cultures of $CD4^+CD25^+$ regulatory T-cells (T_{reg}) and $CD4^+CD25^-$ responder cells revealed T_{reg} from ConA-tolerant mice being more effective in supressing polyclonal T-cell responses than T_{reg} from control mice. Moreover, cellular immune therapy with $CD4^+CD25^+$ cells prevented

ConA-induced liver injury, with higher protection by T_{reg} from ConA-tolerized mice (112). Taken together, these findings indicated that T_{reg} response and activation is paramount to tolerance development towards immune mediated liver damage and IL-6 has an important role in regulatory T-cell development and subsequent protection.

4.5 Outlook

In this work the impact of IL-6 classic- and IL-6 trans-signaling during ConA immune-mediated hepatitis was investigated. It was found that classic IL-6 signaling strongly contributes to the onset of liver damage during the initial phases of ConA immune-mediated liver injury and inflammation, whereas inhibition of IL-6 trans-signaling was found to be not relevant in this process. However, IL-6 was found to have paradoxical effects within this model, since IL-6 pretreated mice were protected from liver damage likely due to altered neutrophil characteristics when released by endogenous IL-6 after ConA challenge. In addition to classic homeostatic granulocyte production mediated by G-CSF and GM-CSF, an independent pathway stimulated by IL-6 trans-signaling has been shown to contribute to emergency granulopoiesis (138). In future experiments, it will therefore be interesting to analyze whether inhibition of IL-6 trans-signaling by sgp130Fc might have an impact in the observed neutrophilia after IL-6 pretreatment. Furthermore, diseases mimicked by ConA hepatitis such as autoimmune and viral hepatitis are long-term diseases. It would therefore be of interest to investigate the impact of permanently high sgp130Fc serum levels on the onset and perpetuation of these hepatic disorders, since in mice these diseases usually develop over a longer time period and would require the long-term administration of exogenous sgp130Fc protein. Therefore, continous inhibition of IL-6-trans-signaling in sgp130Fc transgenic animals will help to clarify the role of sIL-6R-mediated signaling in the bi-phasic release of neutrophils and altered characteristics of neutrophils, contributing to development of immune-mediated liver damage. Future experiments with regulatory T-cells in IL-6 deficient and transgenic sgp130Fc overexpressing mice will additionally yield more

information about IL-6 signaling pathways in tolerance induction observed in ConA- and viral-induced hepatitis.

5 Summary

Interleukin-6 (IL-6) is a pleiotropic cytokine with various cellular functions including induction of acute phase proteins in the liver, stimulation of lymphocytes and control of regenerative processes including wound healing and liver regeneration. Prominent infiltration of lymphocytes into the liver is a hallmark for acute and chronic hepatitis. Liver injury induced by intravenous injection of the mitogenic plant lectin Concanavalin A (ConA) is considered as a murine model of T-cell mediated hepatitis accompanied by up-regulation of proinflammatory cytokines, occurrence of $CD4^+$ T-cells in hepatic lesions but also by the involvement of an activation of resident hepatic NKT, Kupffer cells and neutrophils.

In this study, the role of classic IL-6 signaling and IL-6 trans-signaling during ConA-mediated hepatitis was investigated using IL-6 deficient mice and mice either injected with anti-IL6 mAbs or sgp130Fc. Application of ConA induced liver injury in mice with massive hepatocyte necrosis and neutrophil infiltration. Surprisingly, IL-6 deficient mice and mice treated with an anti-IL-6 mAb but not with sgp130Fc showed reduced ConA induced liver injury. This was accompanied by a diminished increase of circulating and liver infiltrating neutrophils, by an upregulation of the hepatoprotective C-C chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1), and by a downregulation of the hepatodestructive cytokine IL-4, but by unchanged levels of the neutrophil attracting chemokine KC. IL-6 has a dual role in ConA-induced hepatitis, which is hepatoprotective when IL-6 is injected before ConA administration and hepatodestructive if applicated after ConA administration. A single IL-6 injection to mice resulted in release of neutrophils to the circulation. These findings showed that abrogated classic IL-6-signaling in ConA mediated hepatitis exhibited liver-protective effects, which were at least in part caused by the failure to mount an efficient neutrophilia. 8 days after a first ConA injection, mice became tolerant against ConA restimulation. 24 h after a second ConA-challenge IL-6 deficient mice developed severe liver damage accompanied by increased $CD4^+$ T-cell activation compared to completely protected wild-type animals. These data confirmed a dual mode of IL-6 action during ConA-induced hepatitis pointing to early harmful effects mediated by extensive neutrophilia and long term protective effects by an altered T-cell activation pattern.

6 Zusammenfassung

Interleukin-6 (IL-6) ist ein pleiotropes Cytokin mit verschiedenen zellulären Funktionen wie der Induktion von Akut-Phase Proteinen in der Leber, Stimulation von Lymphozyten und ist außerdem bei der Regulierung regenerativer Prozesse wie der Wundheilung und Leberregeneration beteiligt. Vermehrte Lymphozyteneinwanderung in die Leber ist ein Kennzeichen akuter und chronischer Hepatitis. Intravenöse Injektion des Pflanzenlektins Concanavalin A (ConA) führt zu erhöhter Ausschüttung proinflammatorischer Cytokine, vermehrtem Auftreten von $CD4^+$ T-Zellen in entstehenden Leberlesionen, Aktivierung von NKT Zellen, Kupffer Zellen, neutrophilen Granulozyten und gilt als Mausmodell einer T-Zell vermittelten Hepatitis.

In dieser Arbeit wurde unter Verwendung IL-6 defizienter Mäuse, anti-IL-6 Antikörper injizierter Mäuse und sgp130Fc injizierter Mäuse die Rolle von klassischem IL-6 *signaling* und IL-6 *trans-signaling* in der ConA vermittelten Hepatitis untersucht. Die Applikation von ConA führte zu einer Schädigung der Leber mit massiver Nekrose und Einwanderung von Neutrophilen. IL-6 defiziente und mit anti-IL-6 Antikörpern behandelte Mäuse wiesen im Gegensatz zu sgp130Fc behandelten Tieren reduzierte Leberschädigung auf. Zudem wurde ein verminderter Anstieg von Neutrophilen im Blutkreislauf und der Leber, eine Hochregulierung des hepatoprotektiven monocyte chemoattractant protein-1 (MCP-1), eine Herunterregulierung des leberschädigenden Cytokins IL-4, aber keine Veränderung des Neutrophilen anziehenden Chemokins KC gefunden. IL-6 spielt eine duale Rolle während der ConA vermittelten Hepatitis. IL-6 Injektion vor ConA Applikation schützt die Leber, während eine IL-6 Injektion nach ConA Applikation die Leber schädigt. Eine IL-6 Injektion führte zudem zu einer erhöhten Neutrophilenzahl im Blutkreislauf. Die Ergebnisse zeigten, dass unterbrochenes klassisches IL-6 *signaling* während ConA immunvermittelter Hepatitis leberschützende Effekte aufwies, die zumindest teilweise auf eine Störung der effizienten Freisetzung von Neutrophilen in den Kreislauf zurückzuführen ist. Mäuse bilden 8 Tage nach einer ersten ConA Injektion Toleranz gegenüber einer erneuten Stimulierung mit ConA aus. Im Vergleich zu geschützten wildtyp Mäusen entwickelten IL-6 defiziente erneut einen massiven Leberschaden der 24 Stunden nach ConA-Restimulation durch erhöhte Aktivierung von $CD4^+$ T-Zellen gekennzeichnet war. Diese Resultate zeigen die duale Wirkungsweise von IL-6 während ConA induzierter Hepatitis mit anfänglich schädigenden und langfristig schützenden Effekten.

7 References

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8 Appendix

8.1 Abbrevations

Ab	Antibody
ADAM	A disintegrin and metalloproteinase
AIH	Autoimmune hepatitis
ALT	Alanine aminotransferase
ANA	Antinuclear antibody
APC	Allophycocyanin
APS	Ammonium peroxodisulfate
AST	Aspartate aminotransferase
BCA	Bicinchoninic acid
B-cell	Bone marrow derived lymphocyte-cell
Bcl-2	B-cell lymphoma-2
Bcl-X_L	B-cell lymphoma-extra large
BSA	Bovine serum albumine
BSF-3	B-cell stimulatory factor 3
CCL2	Chemokine (C-C motif) ligand-2
CCl4	Carbontetrachloride
CD	Cluster of differentiation
CINC-1	Cytokine-induced neutrophil chemoattractant-1
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
ConA	Concanavalin A
CRP	C-reactive protein
CSF	Colony stimulating factor
CT-1	Cardiotrophin-1
CXCL1	Chemokine (C-X-C motif) ligand-1
Cy7	Cyanine dye 7
d	Days

DCs	Dendritic cells
DMSO	Dimethylsulfoxide
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal regulated kinase
EtOH	Ethanol
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
Fig	Figure
FITC	Fluorescein isothiocyanate
FLIP	FLICE-inhibitory protein
FSC	Forward scatter
g	g-force
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte/macrophage -colony stimulating factor
gp130	Glycoprotein 130
h	Hours
H&E	Hematoxylin & eosin
HIL-6	Hyper-IL-6
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
i.p.-injection	Intraperitoneal-injection
i.v.-injection	Intravenous-injection
ICAM	Intercellular adhesion molecule
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IL-6R	IL-6 receptor
IVC	Individually ventilated cages
JAK	Janus kinase

KC	Keratinocyte chemoattractant
kD	Kilodalton
L	liter
LIF	Leukemia inhibitory factor
LKM	Liver/kidney microsomal antibody
LPS	Lipopolysaccharide
Ly6	Lymphocyte antigen 6
µg	microgram
µl	microliter
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
mg	milligramm
MHC	Major histocompatibility complex
min	Minutes
MIP-2	Macrophage-inflammatory protein-2
ml	milliliter
mM	millimolar
MNC	Mononuclear cells
MOG	Myelin oligodendrocyte glycoprotein
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
ng	nanogramm
NK	Natural killer
NKT	Natural killer T-cell
nm	nanometer
NNT-1	New neurotrophin-1
NP	Neuropoietin
NPN	New neuropoietin
OSM	Oncostatin M
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PBS-T	PBS-Tween
PE	Phycoerythrin
PMN	Polymorphonuclear
POD	peroxidase
pSTAT3	Phospho-STAT3
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SAA	Serum amyloid A
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sgp130	Soluble gp130
sIL-6R	Soluble IL-6R
SMA	Anti-smooth muscle actin antibody
SSC	Side scatter
STAT	Signal transducers and activators of transcription
TACE	Tumor necrosis factor-α converting enzyme
TBS	Tris buffered saline
TBS-T	TBS-Tween
T-cell	Thymus derived lymphocytic-cell
TEMED	N,N,N',N'-Tetramethylethylenediamine
T_h-cell	T-helper cell
TNF	Tumor necrosis factor
TNFR	TNF receptor
T_{reg}	Regulartory T-cell
U	Units
V	Volt
v/v	Volume per Volume
VCAM	Vascular adhesion molecule
w/v	Weight per Volume

8.2 Publications

Malchow S., W. Thaiss, N. Jänner, J. Gewiese, C. Garbers, K. Yamamoto, S. Rose-John and J. Scheller, *Classic IL-6 signaling but not IL-6 trans-signaling controls proinflammatory neutrophilia during ConA-induced hepatitis*. Submitted to J Hepatol, March 2010. In revision.

Drucker C., J. Gewiese, **S. Malchow**, J. Scheller and S. Rose-John, *Impact of interleukin-6 classic- and trans-signaling on liver damage and regeneration*. J Autoimmun, 2010. **34**(1): p. 29-37.

Conrad U., I. Plagmann, **S. Malchow**, M. Sack, D. Floss, A. Kruglov, S. Nedospasov, S. Rose-John and J. Scheller, *ELPylated anti-human TNF-therapeutic single-domain antibodies for prevention of lethal septic shock.* P Biotech J, 2010. **8**: p. 1-10.

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Eidesstattliche Erklärung

Hiermit versichere ich, Sven Malchow, an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur mit Hilfe der angegebenen Hilfsmittel und Quellen unter Anleitung meiner akademischen Lehrer angefertigt habe.

Diese Dissertation wurde bisher an keiner anderen Fakultät vorgelegt.

Ich erkläre, kein anderes Promotionsverfahren ohne Erfolg beendet zu haben und dass keine Aberkennung eines bereits erworbenen Doktorgrades vorliegt.

Kiel, den 19.04.2010

Sven Malchow