

**Generation of sgp130Fc transgenic mice:
Inhibition of IL-6-transsignaling *in vivo* impairs the
proper resolution of acute inflammation**

Dissertation

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Der Dekan

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1 Introduction

1.1 Interleukin-6 and its receptors

Interleukin-6 (IL-6) is a pleiotropic cytokine, which stimulates a variety of cells types including B cells, T cells, keratinocytes, neuronal cells, and endothelial cells. It plays an important role in host defense by regulating immune and inflammatory responses. IL-6 was originally identified as a factor inducing immunoglobulin production in B lymphocytes. Moreover, IL-6 induces expression of several acute phase proteins in the liver and functions as a strong hepatocyte-stimulating factor. IL-6 also stimulates thrombopoiesis and regulates bone resorption (112).

IL-6 belongs to the gp130-family of cytokines comprising 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) and IL-27, which all utilize glycoprotein 130 (gp130) as a common signal transducing subunit. Some of these cytokines need additional membrane-bound or soluble cytokine-receptor proteins to induce signaling. IL-6 first binds its membrane-bound α -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 MAPK (mitogen-activated protein kinase) cascade (44).

The mature IL-6 protein is about 20-28 kDa in size, depending on the glycosylation status. It has been shown that IL-6 is both N- and O-glycosylated (100). It adopts the typical four-helix-bundle fold characteristic for most hematopoietic cytokines containing four α -helices, which are arranged in an up-up-down-down topology (41). IL-6 engages its receptors via three different binding epitopes referred to as site I, II and III (Fig. 1). Site I is located at the C-terminus of the AB-loop and Helix D and mediates binding to the IL-6R. Site II is formed by residues of helices A and C, whereas site III consists of amino acids located at the N-terminal part of the AB-loop and the beginning of Helix D (110). The sites II and III are used to engage and recruit gp130, with site III being unique among the gp130-family of cytokines.

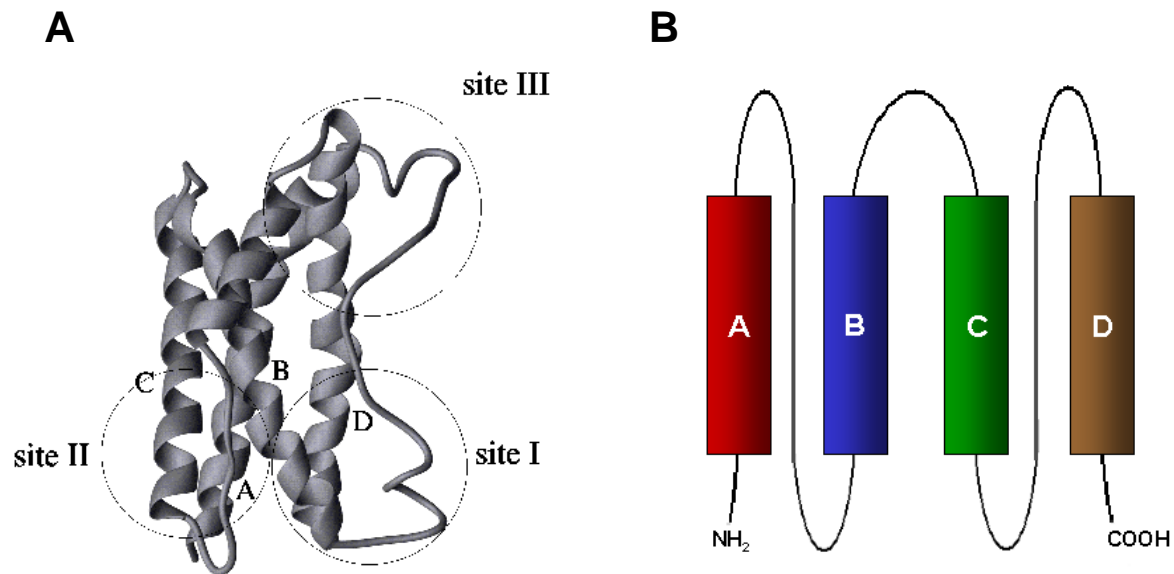


Figure 1: (A) 3D structure of IL-6. The four α -helices (A-D) as well as the binding sites to the IL-6R (site I) and gp130 (sites II and III) are indicated. (B) Typical up-up-down-down topology of IL-6. Site I is located at the C-terminus of the AB-loop and Helix D, site II at helix A and C and site III contains the N-terminal part of the AB-loop and the beginning of Helix D (104).

IL-6R and gp130 are glycosylated type I membrane proteins (extracellular N-terminus, one transmembrane domain). Both receptors are characterized by the presence of one cytokine-binding module (CBM) consisting of two fibronectin-type-III-like domains of which the N-terminal domain contains a set of four conserved cysteine residues and the C-terminal domain a TrpSerXTrpSer (WSXWS) motif (6). IL-6R as well as gp130 contain an IgG-like domain located at the N-terminus of the molecule. Gp130, in contrast to IL-6R, has three additional membrane-proximal fibronectin-type-III-like domains (Fig. 2). The IgG-like domain of gp130 binds to site III of IL-6, whereas the CBM of gp130 interacts with site II. Although the three membrane-proximal domains are not involved in ligand binding, they are necessary for transmitting the signal into the cell, since deletion of these domains leads to signaling incompetent receptors (60). Binding of the IL-6R to the ligand occurs via the CBM (domains 2 and 3), the IgG-like domain is not involved in the engagement of IL-6 and therefore dispensable for biological activity (124). However, the IgG-like domain stabilizes the IL-6R during intracellular trafficking through the secretory pathway (119).

Binding of IL-6 to its receptors is followed by a rapid internalization of the ligand. Within the IL-6R protein no internalization signal has been identified, whereas in gp130 a dileucine-like motif, that is required for receptor-mediated ligand internalization, has been defined (26).

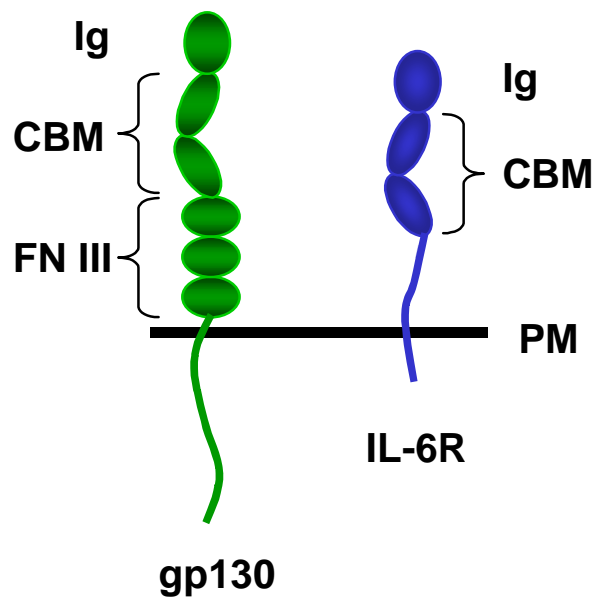


Figure 2: Domain structure of gp130 (green) and IL-6R (blue). Each domain contains roughly 100 amino acids. CBM: Cytokine-binding module. FN III: Fibronectin-type-III-like domains. PM: Plasma membrane. Ig: IgG-like domain.

1.2 IL-6-transsignaling and the designer cytokine Hyper-IL-6

Most soluble cytokine receptors (e.g. IL-1R, TNFR and IL-4R) exhibit antagonistic properties by competing with their membrane-bound counterparts for the respective ligands (25, 39, 120). In contrast, the soluble IL-6R (sIL-6R) acts as an agonist of IL-6 activity. While gp130 is found on all cell types, expression of the IL-6R is largely confined to hepatocytes and some leukocyte subpopulations (112). Cells lacking IL-6R do not respond to IL-6. A naturally occurring soluble form of the IL-6R, which is found in various body fluids of healthy humans (25-35 ng/ml in serum), can render these cells responsive to IL-6. Thereby, sIL-6R binds 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 (Fig. 3). This alternative IL-6 signaling pathway, based on the agonistic properties of the sIL-6R, has been termed transsignaling (54, 98, 99, 104). In addition, IL-6-responsive cells are sensitized by the presence of the sIL-6R protein (89). Among the cell types that can only be activated by IL-6 in the presence of the soluble IL-6R are embryonic stem cells (97), early hematopoietic

progenitor cells (90), neural cells (65-67), smooth muscle cells (56), osteoclasts (113) and endothelial cells (96). In principle, due to the ubiquitous expression of gp130, the IL-6/sIL-6R complex can stimulate virtually all cells of the body. Soluble IL-6R is generated either by limited proteolysis of the membrane-bound protein (78) or by translation from an alternatively spliced mRNA (64). Although the two isoforms of the sIL-6R are structurally and functionally closely related, the cellular release of both forms seems to be differentially regulated (52). In humans, the alternatively spliced variant can be distinguished from the shed form by a novel proximal COOH-terminal sequence (GSRRRGSCGL), which is introduced during the splicing process (48). Shedding of the IL-6 receptor is triggered by different stimuli such as phorbol ester (79), bacterial metalloproteinases and pore-forming toxins (121), C-reactive-protein (53), cholesterol depletion (68) and apoptosis (13). Induced cleavage of the IL-6R is mediated by the metalloprotease TACE (ADAM17), whereas constitutive shedding is dependent on ADAM10 (68).

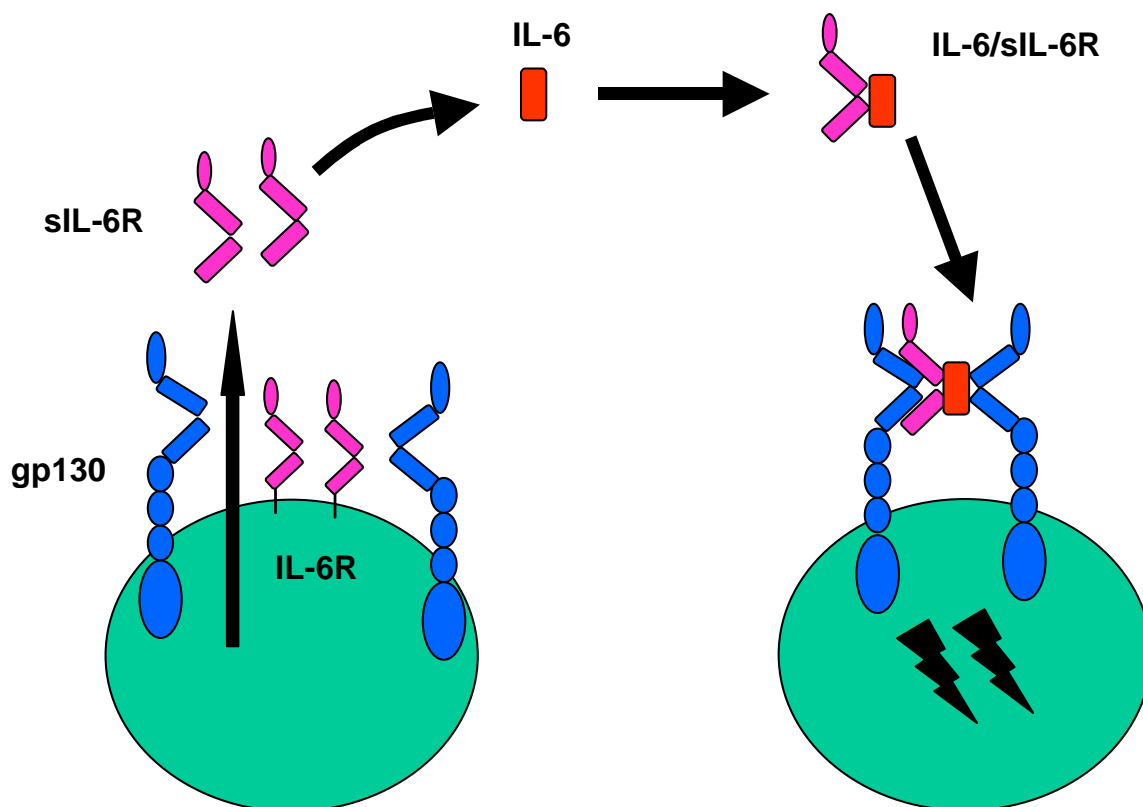


Figure 3: IL-6-transsignaling. A soluble form of the IL-6R (sIL-6R), which is generated via shedding from the membrane surface or alternative splicing, activates cells lacking the membrane-bound IL-6R. Virtually all cells of the body can be stimulated.

In order to mimic IL-6-transsignaling a designer cytokine, designated Hyper-IL-6, was constructed. Hyper-IL-6 is a fusion protein built up of IL-6 and the sIL-6R connected via a flexible peptide linker (Fig. 4), which prevents the natural dissociation-association of the ligand-receptor complex (31). In case of Hyper-IL-6 signaling only occurs via the sIL-6R. It turned out, that Hyper-IL-6 was 100-1000 times more active on gp130-expressing cells (e.g. hematopoietic progenitor cells) than the combination of IL-6 and the sIL-6R (31). Moreover, Hyper-IL-6 has been used to successfully treat acute liver failure in mice and rats (34, 35, 87).

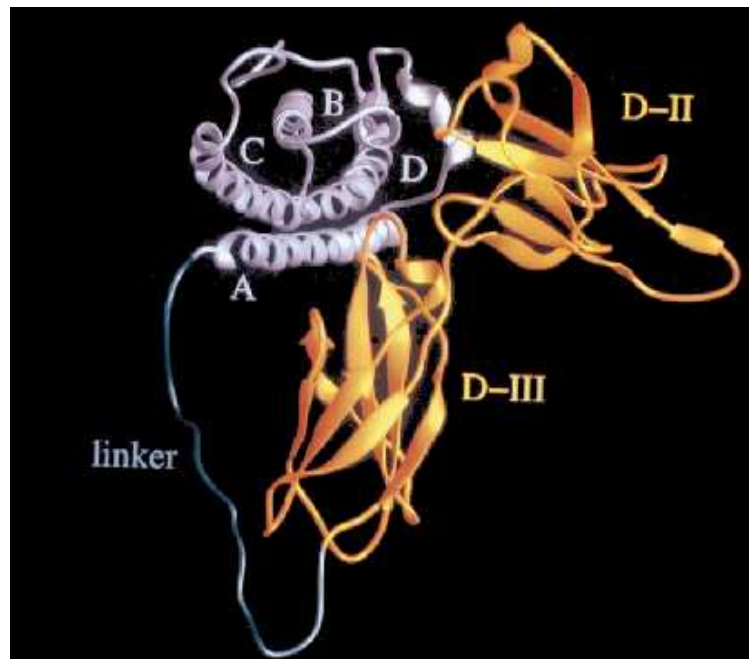


Figure 4: Molecular model of Hyper-IL-6. Hyper-IL-6 consists of IL-6 (grey) and the sIL-6R (yellow) fused by a flexible peptide linker. A, B, C and D are the four α -helices of IL-6, D-II and D-III denote the two ligand binding domains of the IL-6R (31).

In addition to the IL-6R also the second component of the IL-6 receptor complex, gp130, 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 (74, 80, 82, 123) (Fig. 5). It has been concluded that sgp130 is the natural inhibitor of IL-6-transsignaling and that sgp130 acts as a buffer to prevent the stimulation of virtually all cells in the body by the IL-6/sIL-6R complex (55).

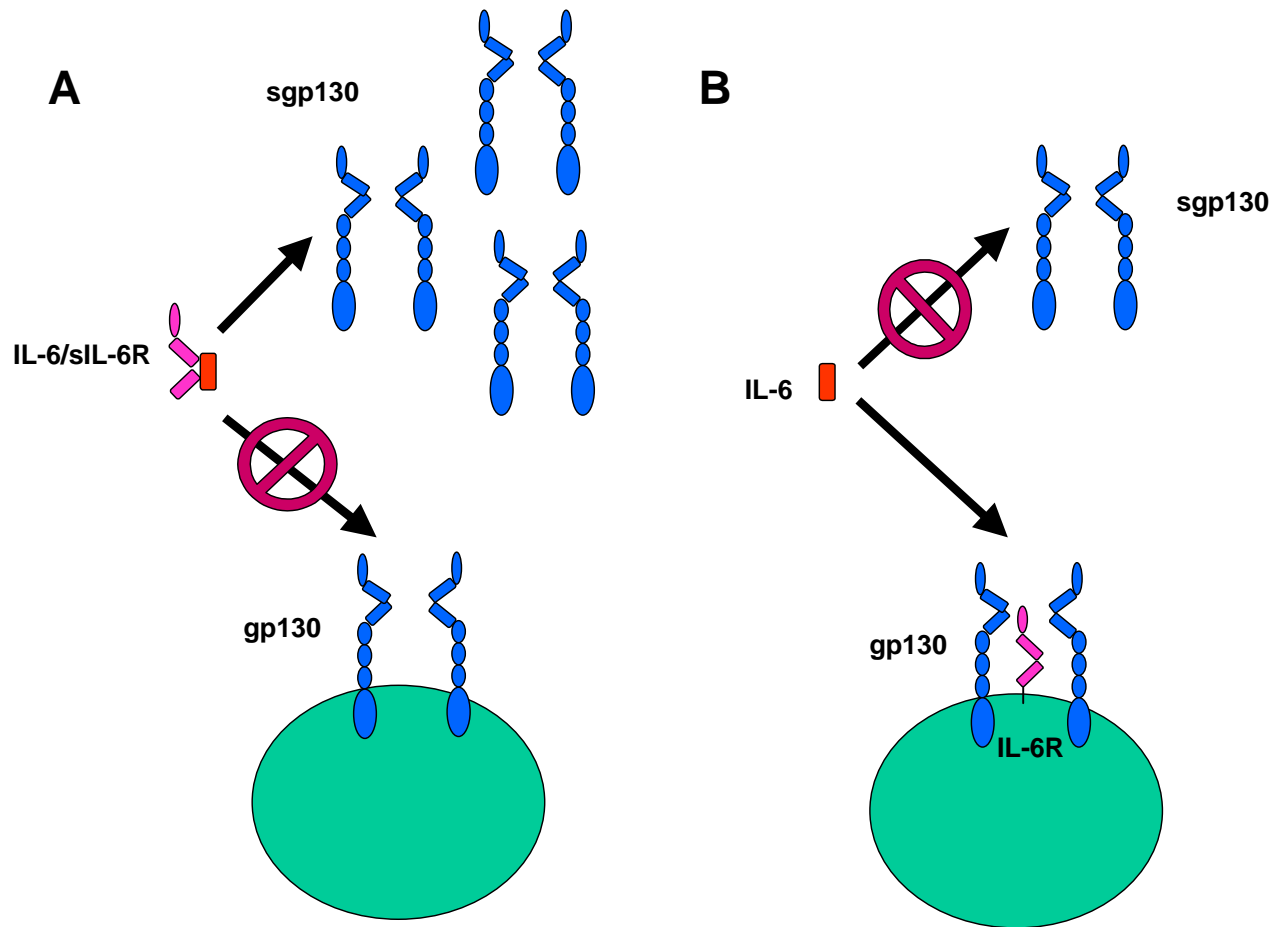


Figure 5: Inhibition of IL-6-transsignaling. Soluble gp130 associates with the IL-6/sIL-6R complex and thereby prevents activation of cells which lack membrane-bound IL-6R. The IL-6/sIL-6R complex can bind to membrane-bound gp130 equally. Therefore a molar excess of sgp130 is needed for competitive inhibition (A). Classical IL-6-signaling remains unaffected, since sgp130 cannot bind to IL-6 in solution (B).

Interestingly, it has been found that sgp130 also blocks LIF- and OSM-signaling although to a much lesser extent (55, 105).

Similar to the sIL-6R, both proteolytic cleavage and alternative splicing have been implicated in the cellular liberation of sgp130 (52, 81). On the other hand, it has been shown that after phorbol ester stimulation proteolytic cleavage of gp130 was considerably less than observed for the IL-6R (76) suggesting that alternative splicing and not shedding is the main mechanism responsible for sgp130 release. Recently, a 50-kDa isoform of sgp130 was identified as an autoantigen in rheumatoid arthritis (114). This alternatively spliced protein

harbours a unique COOH-terminal sequence (NIASF) and a high incidence of serum antibodies against this sequence have been detected in patients suffering from rheumatoid arthritis. Gp130-RAPS (gp130 of the rheumatoid arthritis antigenic peptide-bearing soluble form) has been shown to bind with high affinity to Hyper-IL-6 and to effectively modulate leukocyte migration in a murine peritonitis model (94).

A variant of sgp130 was constructed in our group by fusing the complete extracellular region of gp130 (domains D1-D6) with the Fc-part of a human IgG-antibody (Fig. 6). The resulting protein, called sgp130Fc, 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 (55). Thus, classic IL-6-signaling and IL-6-transsignaling can easily be distinguished with the help of sgp130Fc.

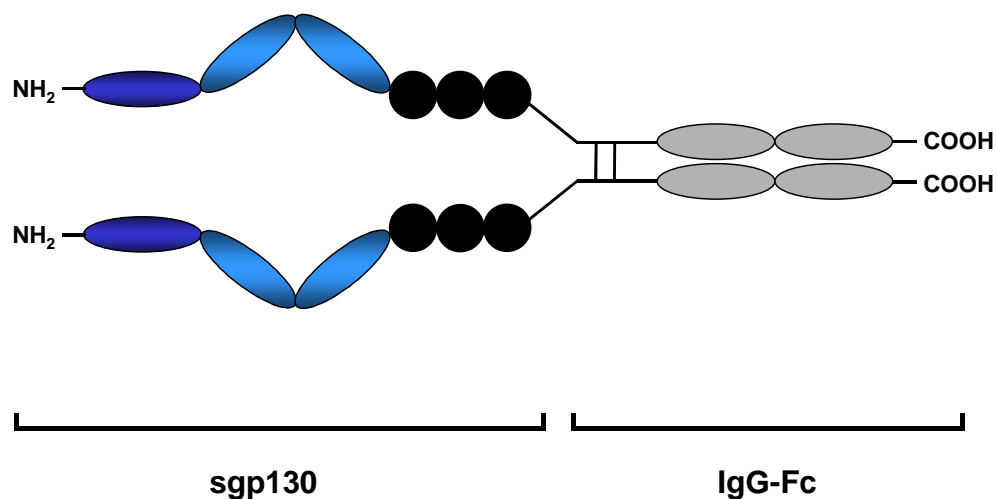


Figure 6: Sgp130Fc is a preformed dimer consisting of the extracellular part of gp130 fused to the Fc portion of a human IgG1 antibody.

1.3 Pathophysiology of IL-6-transsignaling

Elevated levels of sIL-6R have been detected in several inflammatory and neoplastic disorders and it has been shown that IL-6-transsignaling is involved in the pathogenesis as well as in the persistence of these diseases (54).

In inflammatory bowel disease, which is a chronic inflammatory disease of the gastrointestinal tract, sIL-6R mediated signaling contributes to the increased resistance of mucosal T cells against apoptosis. In these cells signal transducer and activator of transcription 3 (STAT-3) as well as the anti-apoptotic genes bcl-2 and bcl_{xl} are highly upregulated. *In vivo* administration of recombinant sgp130Fc leads to T-cell apoptosis and suppression of colitis activity indicating that IL-6-transsignaling accounts for both development and perpetuation of chronic intestinal inflammation (4).

Elevated sIL-6R concentrations have been observed in the joints of patients suffering from arthritis, with the highest levels being detected in the more progressive stages of rheumatoid arthritis (59, 95). In addition, a strong correlation between the infiltration of leukocytes into arthritic joints and increased sIL-6R levels in the synovial fluid was found (23). Induction of arthritis (both antigen-induced and collagen-induced) in IL-6-knockout mice resulted in no clinical signs or in significantly reduced disease (2, 9). Intra-articular administration of Hyper-IL-6, in contrast to IL-6 alone, restored full disease activity in IL-6-knockout mice demonstrating that sIL-6R signaling is involved in the pathogenesis of rheumatoid arthritis. In addition, neutralization of IL-6-transsignaling in wild-type mice using sgp130Fc protein ameliorated the disease (85).

Recent studies have shown that sIL-6R mediated events control leukocyte infiltration in murine models of acute inflammation (49, 96). It was demonstrated in several *in vitro* studies that IL-6 in combination with sIL-6R can activate various cell types to produce a certain subset of chemokines (IL-8, MCP-1 and MCP-3) and adhesion molecules (ICAM-1 and VCAM-1) (49, 56, 58, 73, 96). Later it became evident that the IL-6/sIL-6R complex plays an important role in regulating the switch between the early, predominantly neutrophilic stage of an infection/inflammation and the more sustained mononuclear cell influx (54).

In colon cancer the expression of both IL-6 and sIL-6R is drastically increased (109). IL-6 can promote the growth of colon cancer cells and enhanced IL-6 levels were found to correlate with tumor size (17, 33). In a murine colitis-associated colon carcinoma model, treatment with an anti-IL-6R-antibody as well as sgp130Fc significantly reduced tumor number and size indicating that IL-6-transsignaling promotes colon carcinogenesis in mice (7). Interestingly, the IL-6R is completely lost on epithelial tumor cells most likely due to proteolytic cleavage by the metalloproteinase ADAM17 (8). It turned out that during colon carcinogenesis IL-6-transsignaling is controlled by infiltrating CD4⁺CD8⁺-T lymphocytes via a TGFβ-related mechanism, as transgenic mice overexpressing TGFβ had less tumors and mice with a deficient TGFβ-T cell response showed enhanced tumor development (7).

Furthermore, soluble IL-6R-signaling has also been implicated in the pathogenesis of asthma (28), ileitis, which is a form of intestinal inflammation (72), and multiple myeloma (32, 42).

1.4 Viral IL-6

Viral IL-6 (vIL-6) is encoded by human herpesvirus 8 (HHV 8), which is associated with Kaposi sarcoma (KS) and lymphoproliferative disorders, such as pleural effusion lymphoma and multicentric Castleman's disease (75, 83, 84). The viral homologue shares 25% amino acid identity with human IL-6 and it turned out that both cytokines utilize the gp130 receptor system (12, 45). In contrast to human IL-6, which requires binding to the IL-6R before engaging gp130, vIL-6 can directly associate with gp130 (47, 62, 77).

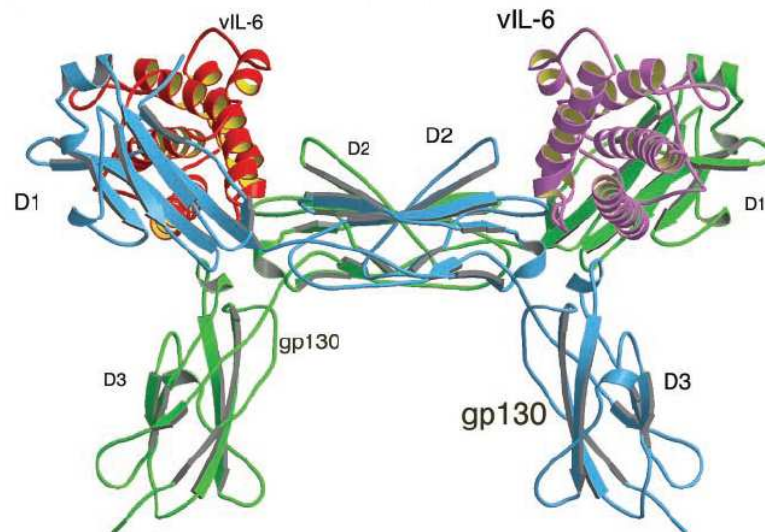


Figure 7: 3D structure of vIL-6 (red and magenta) in complex with the domains D1-D3 of gp130 (blue and green). The signaling complex is a tetramer consisting of two molecules of vIL-6 and gp130 (16).

The solution of the 3D structure of vIL-6 in complex with the extracellular portion of gp130 revealed that two vIL-6 molecules are complexed in a ring-like fashion with two molecules of gp130. The structure also shows that vIL-6 binds gp130 via site II (engages the CBM of the first gp130) and site III (associates with D1 of the second gp130), whereas site I remains

unoccupied (16). So far, vIL-6 is the only naturally occurring cytokine which directly binds and activates gp130. Like the IL-6/sIL-6R complex vIL-6 is able to stimulate virtually all cells in the body and therefore mimicks IL-6-transsignaling (57, 77).

It turned out that vIL-6 effectively blocks a major antiviral immune mechanism induced by IFN signaling. In infected cells IFN- α normally leads to p21 mediated cell cycle arrest and downregulation of membrane-bound IL-6R, thereby rendering the cells unresponsive to the proliferative effects of IL-6. Viral IL-6, which can activate gp130 expressing cells that lack the cognate IL-6R, is still able to mediate signal transduction and activation of these cells by creating an autocrine secretion loop (14).

Analogous to the IL-6/sIL-6R complex, the biological activity of vIL-6 can be completely inhibited by sgp130Fc (77).

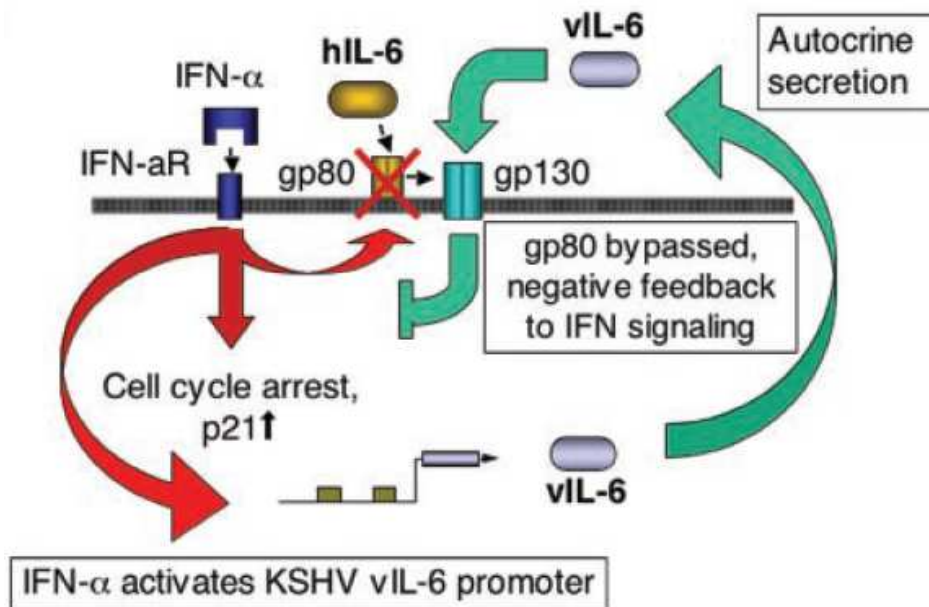


Figure 8: Model for HHV8 vIL-6 mediated inhibition of IFN- α signaling. The vIL-6 promoter is activated by IFN- α , vIL-6 protein is produced allowing infected cells to proliferate, albeit IFN- α leads to IL-6R (gp80) downregulation (14).

1.5 Aim of this work

To investigate the pathophysiological consequences of IL-6-transsignaling *in vivo*, transgenic mice were constructed and characterized, which overexpress sgp130Fc from a liver-specific promoter. High levels of transgenic sgp130Fc could be detected in the serum of these mice and it was demonstrated that the protein was functionally active. A murine model of acute inflammation was employed and it turned out that in sgp130Fc-transgenic mice leukocyte recruitment was profoundly impaired, indicating that IL-6-transsignaling plays an important role in the resolution of inflammation.

Sgp130Fc transgenic animals are therefore an ideal tool to study the pathophysiology of sIL-6R-mediated responses *in vivo*, as they reflect an “IL-6-transsignaling knock out” situation in mice.

2 Material and Methods

2.1 Material

Chemicals:

All chemicals used were from ROTH, SIGMA or MERCK. If it is not mentioned otherwise deionized water (Millipore) was used as solvent.

Bacterial strains and cell lines:

- *E. coli* DH5 α (Invitrogen)
- *E. coli* XL1 blue (Stratagene)
- HepG2, human hepatocarcinoma cell line (ATCC)

Vectors:

- pTZ-pEPCK- β glob.intron : vector used for transgene generation (89)
- p409-sgp130Fc, p409-sgp130Fc/opt : eukaryotic expression vectors

Primer:

- SAA2-forward: 5` - TCA CCA GCC TGG TCT TCT G-3`
- SAA2-reverse: 5` - CTG AGC TAA TAG GAG GAC G-3`
- sgp130Fcopt-forward: 5` - GAG TTC AGA TCC TGC GAC-3`
- sgp130Fcopt-reverse: 5` - TCA CTT GCC AGG AGA CAG-3`

All primers used in this work were purchased from METABION.

Antibiotics:

- Ampicillin 50 µg/ml in H₂O
- Kanamycin 50 µg/ml in H₂O

Media:

- LB-Medium: 5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl
- LB-Agar: 5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl, 15g/l agar
- Cell growth medium: DMEM High Glucose Culture Medium (PAA), 10% FCS, 60 mg/l penicillin, 100 mg/l streptomycin

Enzymes:

All used restriction enzymes and DNA polymerases (Taq, Pfu and T4), as well as T4 DNA Ligase, calf intestine alkaline phosphatase (CIAP) and T4 polynucleotidkinase were purchased from Fermentas.

Enzyme substrates:

- Substrate Reagent Pack (R&D Systems) - 3,3',5,5'-Tetramethylbenzidine, horseradish peroxidase substrate for ELISA
- ECL plus Western blotting detection system (Amersham)

Antibodies:

- Mouse anti-human sgp130 BP-4 (Diaclone), diluted 1:1,000
- Mouse anti-human sIL-6R clone 14-18, 1:10,000 (produced in our laboratory)
- Anti-mouse IgG goat peroxidase conjugate (Pierce), 1:10,000

2 Material and Methods

- Alexa Fluor 488-conjugated anti-mouse Ly-6G and Ly-6C (BD Pharmingen), 1:400 for Flow Cytometry
- APC-conjugated anti-mouse F4/80 (Caltag), 1:400 for Flow Cytometry
- Purified anti-mouse CD16/CD32 (Fc γ III/II Receptor, mouse Fc block, BD Pharmingen), 1:500 for Flow Cytometry
- purified anti-mouse Ly-6GC (BD Pharmingen), 0.1 mg/mouse i.p., for neutrophil depletion in mice
- PE-conjugated anti-mouse B220 (BD Pharmingen), 1:400 for Flow Cytometry
- FITC-conjugated anti-mouse CD3 (BD Pharmingen), 1:200 for Flow Cytometry

Recombinant cytokines:

- Hyper-IL-6 (100 ng/ μ l), prepared and purified as described in (31)
- human IL-6 (100 ng/ μ l), prepared and purified as described in (117)
- human sgp130Fc (100 ng/ μ l), prepared and purified as described in (55)

Molecular markers:

- Protein molecular weight marker (Fermentas)
- Prestained protein molecular weight marker (Fermentas)
- GeneRuler 1 kb DNA Ladder (Fermentas)
- Gene Ruler 100bp Ladder (Fermentas)

Radiochemicals:

Deoxyadenosine-5'-triphosphate, [α -³²P], 3000 Ci/mmol was purchased from MP Biomedicals.

Membranes:

- Hybond-N+ (Amersham), positively charged nylon membran
- Hybond-P (Amersham), PVDF membrane

Kits:

- NucleoSpin RNA II (Macherey-Nagel)
- QIAquick PCR Purification Kit (Qiagen)
- QIAquick Gel Extraction Kit (Qiagen)
- NucleoBond PC 100 DNA PurificationKit (Macherey-Nagel)
- DuoSet human gp130 ELISA Kit (R&D Systems)
- Duo Set mouse IL-6 ELISA Kit (R&D Systems)
- DuoSet mouse KC ELISA Kit (R&D Systems)
- Ready-SET-Go! mouse MCP-1 ELISA Kit (eBiosciences)
- Mouse IL-6R ELISA Kit (developed by S. Jones, Cardiff)
- Prime-It II Random Primer Labeling Kit (Stratagene)

Buffers:

- TBS: 50 mM Tris-HCl pH 7.6, 200 mM NaCl
- TBS-T: 50 mM Tris-HCl pH 7.6, 200 mM NaCl, 0.05% Tween-20
- PBS: 250 mM NaCl, 20 mM KCl, 48 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.4
- PBS-T: 250 mM NaCl, 20 mM KCl, 48 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.4,
0.05% Tween-20
- 0.5x TBE: 45 mM Tris-borate, 1 mM EDTA pH 8.0
- Flow Cytometry antibody buffer: 1x PBS, 1% BSA, N₃Na (1g/l)

2 Material and Methods

- 20x SSC: 3 M NaCl, 0.3 M sodium citrate pH 7.0
- Southern/Northern blot hybridization solution (18): 0.5 M Na₂HPO₄, 7% SDS, 1 mM EDTA, 1% BSA, pH 7.2
- Southern blot denaturation solution: 1.5 M NaCl, 0.5 M NaOH
- Southern blot neutralization solution : 1.5 M NaCl, 1 M Tris-HCl pH 7.0
- Southern/Northern blot stripping buffer: 15 mM NaCl, 0.1x SSC, 1% SDS
- 5x MOPS : 0.1 M MOPS pH 7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0
- 2x SDS gel-loading buffer (61): 100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol
- 10%-SDS-electrophoresis separating gel: 4.05 ml H₂O, 2.5 ml 1.5 M Tris-HCl pH 8.8, 100 µl 10% SDS, 3.3 ml 30% acrylamid/0.8% bisacrylamid, 50 µl 10% APS, 5 µl TEMED
- 4%-SDS-electrophoresis stacking gel: 3.725 ml H₂O, 0.625 ml 0.5 M Tris-HCl pH 6.8, 50 µl 10% SDS, 0.67 ml 30% acrylamid/0.8% bisacrylamid, 25 µl 10% APS, 5 µl TEMED
- Tris-glycine SDS-electrophoresis buffer: 25 mM Tris-base, 250 mM glycine pH 8.3, 0.1% SDS
- Coomassie staining solution: 1g/l Coomassie R250, 40% methanol, 10% acetic acid
- Destaining solution: 40% methanol, 10% acetic acid
- Western blot transfer buffer: 39 mM glycine, 48 mM Tris-base, 20% methanol
- Western blot stripping buffer: 2% SDS, 62.5 mM Tris-base, 100 mM β-mercaptoethanol
- 10x IP-buffer: 0.25 M Tris-HCl pH 7.5, 5% Triton-X100, 5% NP-40
- IP-Washing buffer A: 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2 % NP-40, 2 mM EDTA

2 Material and Methods

- IP-Washing buffer B: 10 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.2 % NP-40, 2 mM EDTA
- Tail buffer: 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 20 mM NaCl, 1% SDS, 100 µg/ml Proteinase K

Animals:

- Wildtype mice: C57BL/6 (Charles River, Germany)
- Founder mice: B6D2F1 (Biotechnologielabor, Universität Heidelberg)

Animal food:

The standard feed as well as the protein rich/carbohydrate low diet was obtained from SSNIFF.

Standard :	crude protein	19 %
	crude fat	3.30 %
	crude fibre	4.90 %
	crude ash	6.70 %

Protein rich/ carbohydrate low:	casein	64 %
	crude fibre	22 %
	vegetable oil	11 %
	brewer`s yeast	2 %

Additives: Vitamin A: 15.000 IE/kg; Vitamin D3: 1.000 IE/kg; Vitamin E: 100 mg/kg;

Cu: 5 mg/kg

2.2 Methods

2.2.1 Agarose gel electrophoresis

DNA gel electrophoresis was performed at 100V for 45 min in 0.5x TBE buffer. DNA-Fragments >700 bp were separated on 1% agarose gels, whereas fragments <700 bp were separated on 2% agarose gels. DNA was stained with Ethidiumbromide (0.5 µg/ml) and visualized under UV-light (312 nm). If necessary, the desired fragments were cut out with a sterile scalpel and DNA was isolated using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer`s instructions.

2.2.2 Cloning

Plasmids were isolated either by Alkaline lysis according to Sambrook et al. (103) or, if high purity or quantity was needed, using the NucleoBond PC 100 DNA Purification Kit (Macherey-Nagel). Restriction digests and DNA ligations were carried out using the appropriate buffers and enzymes from Fermentas according to the manufacturer`s instructions.

2.2.3 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction is an elegant and effective method to selectively amplify a desired target DNA from only a few molecules. If the PCR product was subcloned, Pfu polymerase (Fermentas) was employed, which exhibits an intrinsic proof reading activity, otherwise Taq Polymerase (Fermentas) was used.

Standard PCR reaction settings:	5x Taq Polymerase buffer	5 µl
	MgCl ₂ (25 mM)	4 µl
	dNTPs (10 mM)	1 µl
	Primer A (100 pmol)	0.5 µl
	Primer B (100 pmol)	0.5 µl
	Taq-Polymerase (2.5 U/µl)	1 µl
	DNA Template	10-100 ng
	H ₂ O	ad 50 µl

Standard PCR programm :	94°C	2 min		initial denaturation step
	94°C	30 sec	} x30	denaturation
	55°C	30 sec		primer annealing
	72°C	1 min		elongation (1 min/1 kb)
	72°C	5 min		final elongation step

If necessary, the standard PCR program was modified, depending on the size and frequency of the amplified fragment, and on the T_m of the primers.

2.2.4 RT-PCR

RT-PCR is a PCR amplification of a product from the reverse transcription (RT) reaction, whereby a template mRNA is translated into a single-stranded cDNA. RevertAid M-MuLV Reverse Transcriptase from Fermentas was employed according to the manufacturer's instructions. The reactions were performed using oligo(dT) primer targeting the 3' poly(A) mRNA tail. The PCR settings were described above.

2.2.5 Transformation of bacteria

Plasmid DNA or ligations were transformed via heatshock. In brief, 50 μ l stored chemically competent bacterial cells DH5 α were thawed on ice and added to the chilled DNA samples, mixed and incubated for 5 min. Transformation was achieved at 42°C for 60 sec. After an additional 5 min incubation step on ice, 300 μ l LB-Medium without antibiotic were added to the sample and incubated at 37°C for 1 h under continuous shaking to allow the bacteria to recover. The transformed bacterial cells were then plated onto LB agar containing the selective antibiotic and grown overnight at 37°C.

2.2.6 Transient transfection of HepG2 cells with DEAE Dextran

HepG2 cells were splitted and grown to be 70-80% confluent in DMEM High Glucose Culture Medium (PAA) supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C with 5% CO₂ in a humidified atmosphere. Before transfection, the culture medium was changed to medium containing 75 µM Chloroquin. For one 10 cm cell culture dish, 5 µg of the appropriate plasmid DNA was thoroughly mixed with 435 µl Chloroquin-containing medium and 65 µl DEAE-Dextran (4 mg/ml). The mixture was dropwise added to the cells followed by 4-6 h incubation at 37°C. Then the culture medium was removed and the cells were treated with medium containing 10% DMSO for 7 min at room temperature. Subsequently the cells were washed with PBS and new medium was added. The next day, the medium was exchanged again.

2.2.7 Serum/Plasma preparation from whole mouse blood

Whole mouse blood was collected either by tail bleeding or cardiac puncture. The blood was allowed to clot for 2-3 h at room temperature or over night at 4°C. Afterwards, the serum was seperated by centrifugation (10 min, 2,600 rpm, room temperature) and then stored at -20°C. If Plasma was needed, 10 µl 0.5 M EDTA were added to the blood to prevent clotting. Plasma was obtained by centrigation (10 min, 13,000 rpm, 4°C) and stored at -20°C.

2.2.8 Immunoprecipitation with Protein A Sepharose

Protein A binds to the Fc region of immunoglobulins. Sgp130Fc is a fusion protein containing the Fc portion of a human IgG1 antibody and can therefore be precipitated with Protein-A-Sepharose. To precipitate sgp130Fc from transfected HepG2 cell supernatant 10 ml conditioned medium was cleared from cell debris by centrifugation (5 min at 1,000 rpm) and ultrafiltration (0.22 µm) and incubated with 50 µl Protein-A-Sepharose (50% slurry in PBS) over night rolling at 4°C. On the next day the Protein-A-Sepharose was washed twice with 1 ml IP-Washing buffer A and twice with 1 ml IP-Washing buffer B. After addition of 50 µl 2x

SDS gel-loading buffer and heating for 5 min at 95°C the sample was loaded on a 10% polyacrylamide gel. Sgp130Fc was detected by Western blotting with the mAB BP-4.

For the precipitation of Hyper-IL-6 with transgenic sgp130Fc, 40 µl mouse serum was incubated with 900 µl 1x PBS, 100 µl 10x IP-buffer and 0.5 µg Hyper-IL-6 at 4°C over night under continuous rolling. On the next day, 30 µl Protein-A-Sepharose was added to pull down sgp130Fc and incubated for 2 h at 4°C, again under continuous rolling. The Protein-A-Sepharose was washed and treated as described above. Detection of the co-precipitated Hyper-IL-6 occurred with mAB sIL-6R clone 14-18, which was produced in our laboratory. Transgenic sgp130Fc was precipitated from mouse serum by mixing 50 µl serum with 900 µl 1x PBS, 100 µl 10x IP-buffer and 30 µl Protein-A-Sepharose followed by incubation at 4°C over night. Treatment of the Protein-A-Sepharose and detection of the protein proceeded as described above.

2.2.9 Western blot analysis

The samples containing either sgp130Fc or Hyper-IL-6 were separated on 10% polyacrylamide gels for 1.5 h at 200 V. Protein Transfer onto a PVDF membrane was performed at 15 V for 90 min. The membrane was blocked in 6% dry, low fat milk/TBS for 1 h at room temperature followed by over night incubation with the primary antibody diluted in 1% milk/TBS-T at 4°C. After intensive washing with TBS-T the membrane was incubated with the secondary POD conjugated antibody at room temperature for 1 h. Afterwards the membrane was washed repeatedly (3x 15 min TBS-T and finally 5x5 min TBS) and the peroxidase substrate ECL(+) was applied. X-Ray film was exposed to the chemiluminescent signal.

2.2.10 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISAs for KC, murine MCP-1, murine IL-6, murine IL-6R and human sgp130 were performed according to the manufacturer's instructions. For the human sgp130 ELISA recombinant sgp130Fc was used as a standard with the highest concentration being 10 ng/ml. Standard and serum samples were diluted with 10% FCS/1x PBS.

2.2.11 Southern blot analysis of genomic mouse DNA

0.5 cm of the mouse tail was cut with steril scissors at the age of 3-4 weeks. The tail was shaken at 55°C over night in Tail buffer containing Proteinase K until the tissue was completely dissolved. After two phenol/chloroform/isoamylalcohol (25:24:1) purification steps and one chloroform extraction the DNA was precipitated with 96% Ethanol. The DNA sample was centrifuged (20 min, 13,000 rpm) at room temperature, the pellet was washed once with 70% Ethanol and thereafter air dried for 1-2 h under a fume hood. DNA was dissolved in 100 µl H₂O over night at room temperature. The concentration of genomic DNA was measured by UV-spectrophotometry. 5-10 µg DNA were digested with the restriction endonuclease NcoI (“first” generation of sgp130Fc transgenic mice) or XhoI (“second” generation of codon-optimized sgp130Fc transgenic mice) and separated on a 1% agarose gel for 90 min at 100 V. The gel was treated with 0.25 M HCL for 15 min, 2x 15 min with southern blot denaturation solution and finally with southern blot neutralization solution for 10 min. DNA fragments were transferred from the agarose gel to a positively charged nylon membrane by Southern blotting (111). The capillary blot was assembled and performed according to Sambrook et al. (103), resulting in immobilization of the DNA fragments on the nylon membrane. Additionally, DNA was crosslinked to the membrane by UV radiation. Thereupon, the nylon membrane was hybridized with a α -³²P-dATP-labeled probe (consisting of a XbaI-fragment comprising the entire sgp130Fc cDNA for the „first“ generation or a XhoI-fragment covering the entire codon-optimized sgp130Fc cDNA for the “second” generation of sgp130Fc transgenic mice) in prewarmed southern blot hybridization solution under continuous rolling at 65°C over night. The radioactive labeled probe was generated with the Prime-It II Random Primer Labeling Kit (Stratagene) according to the manufacturer`s instructions. To remove excess probe, the membrane was repeatedly washed with 2x SSC, 0.1% SDS (moderate stringency) and 0.1x SSC, 0.1% SDS (high stringency) at 65°C under rolling conditions. The target sequence was visualized on X-ray film by autoradiography. The same nylon membrane could be used for several consecutive hybridizations with different probes. For successful removal of probes the filter was treated with a 1% SDS solution for 30 min at 80°C.

2.2.12 Northern blot analysis

Appropriate mouse organs were excised and immediately frozen in liquid nitrogen to prevent degradation of the RNA by almost omnipresent RNases. Thereafter, the frozen mouse tissue was disrupted by grinding with a pestle and mortar in the presence of liquid nitrogen. The homogenized organs were subsequently processed or kept at -70°C for long-term storage. It was taken care that the organs did not thaw during or after the grinding. Total RNA was isolated with the NucleoSpin RNA II Kit (Macherey-Nagel) and the RNA concentration was determined by UV-spectrophotometry. The RNA was separated under denaturing conditions on a 1% agarose gel containing 7% formaldehyde. Electrophoresis was carried out in 1x MOPS buffer for 80 min at 100 V. To avoid RNA degradation all buffers and solutions were treated with 0.05% diethylpyrocarbonate (DEPC) over night at 37°C and subsequently autoclaved for 20 min (DEPC derivitizes histidine residues and is therefore an effective method to inactivate RNases). The gel was soaked over night in DEPC H_2O under gentle agitation to remove formaldehyde. Total RNA was visualized under UV-light. Intact RNA should yield two sharp rRNA bands, with the 28S band being about twice as intense as the 18S band. Before assembling the blot the gel was equilibrated in 20x SSC for 45 min under cautious shaking. The physical set-up for Northern transfer was identical to that for Southern transfer (see above) except that 20x SSC was used as transfer buffer. RNA was fixed on the filter through UV-crosslinking and then hybridized with a radioactive labeled DNA probe. The probe was prepared with the Prime-It II Random Primer Labeling Kit (Stratagene) corresponding to the manufacturer's instructions based upon a purified PCR-Fragment of the mouse SAA2 gene or a XhoI restriction fragment comprising the entire sgp130Fc/opt cDNA, depending on the purpose of the experiment. Hybridization and washing of the filter was achieved as described for Southern blotting. The desired target RNA was visualized on X-ray film by autoradiography.

2.2.13 Murine air pouch model of acute inflammation

Animals used throughout the air pouch experiments were 8-10 weeks old male C57BL/6 mice (Charles River), homozygous sgp130Fc-transgenic mice of the lines opt2 and opt3 (with sgp130Fc serum levels above $25\ \mu\text{g/ml}$) or their wildtype littermates. The air pouch model of local inflammation was performed according to Edwards et al. (29). Mice were anesthetized

2 Material and Methods

with ether and subcutaneous dorsal pouches were generated by injection of 6 ml sterile air. After three days the pouches were reinjected with 4 ml of air. On day 6, 1 ml of a suspension of 1% Carrageenan (SIGMA) in sterile PBS (PAA) was injected into the cavity, whereas corresponding control animals received sterile PBS. Carrageenan is composed of mainly sulfated polysaccharides, which are generally extracted from seaweed. Injection of Carrageenan into air pouches of rats and mice is a well established disease model of acute, local inflammation (22, 29, 107). At different time points following Carrageenan challenge, mice were sacrificed and the pouches were washed with 3 ml ice-cold PBS. Exudates were collected and the volume was recorded. Total cell number was counted with a Neubauer cell-counter chamber. The remaining lavage fluid was centrifuged at 5,000 rpm for 10 min at 4° C, and the supernatant was stored at -20°C until analyzed by ELISA. Levels of chemokines and cytokines/soluble cytokine receptors were determined using commercial ELISA Kits. Aliquots of the exudate containing 2×10^5 cells were used for Flow Cytometry analysis, whereby the mABs Ly6GC (BD Pharmingen), F4/80 (Caltag), CD3 (BD Pharmingen) and B220 (BD Pharmingen) were employed to count infiltrating neutrophils, macrophages, T cells and B cells, respectively.

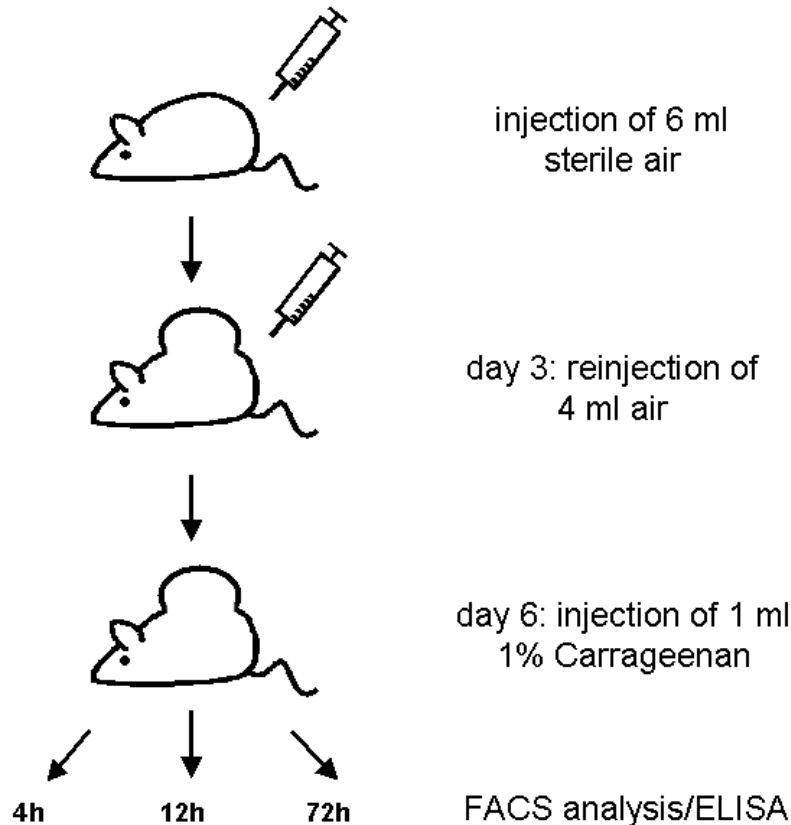


Figure 9: Schematic representation of the air pouch time schedule. At different time points after Carrageenan injection the pouch was washed with 3 ml PBS and the exudate was analyzed by FACS or ELISA.

2.2.14 Neutrophil depletion

8-10 weeks old male C57BL/6 mice with 6-day-old air pouches received an i.p. injection of 100 µg purified anti-mouse Ly6GC mAB (BD Pharmingen) 18 h prior to 1% Carrageenan challenge. The exudates were collected 24 h and 72 h after Carrageenan injection and processed and analyzed as described above.

2.2.15 Analysis of the air pouch exudate by Flow Cytometry

The cells in the lavage fluid were counted with a Neubauer cell-counter chamber and 2×10^5 cells were used for Flow Cytometry analysis. Cells were washed once with 500 µl Flow Cytometry antibody buffer and centrifuged for 5 min at 1,000 rpm. To block Fc-receptors on neutrophils and macrophages the cell suspension was incubated for 5 min with Mouse BD Fc Block CD 16/32 mAB (1:500 diluted in 100 µl Flow Cytometry antibody buffer). After adding antibodies all incubation and centrifugation steps were carried out at 4°C. Since Mouse BD Fc Block does not need to be washed off before staining, the cells were subsequently treated with the fluorescence-coupled mABs against Ly6GC or F4/80 for 1 h in order to specifically detect neutrophils and macrophages, respectively (1:400). B cells and T cells were stained in such a way with fluorescence-coupled mABs against B220 (CD45R) and CD3, respectively (1:400 and 1:200). To prevent bleaching of the antibody-conjugated fluorescent dye the reaction tubes were protected from direct light. Cells were washed once with 500 µl Flow Cytometry antibody buffer followed by centrifugation for 5 min at 1,000 rpm and finally resuspended in 1 ml antibody buffer. Afterwards, the stained cells were analyzed by Flow Cytometry.

The forward scatter/side scatter (FSC/SSC) plot revealed that the cellular distribution in the air pouch roughly resembles the situation observed in peripheral blood. Both cellular debris and Carrageenan particles could be excluded from the measurement by setting an appropriate gate, being sure that only intact cells were applied for Flow Cytometry analysis (Fig. 10). In general, data were acquired from 10,000 gated events per sample.

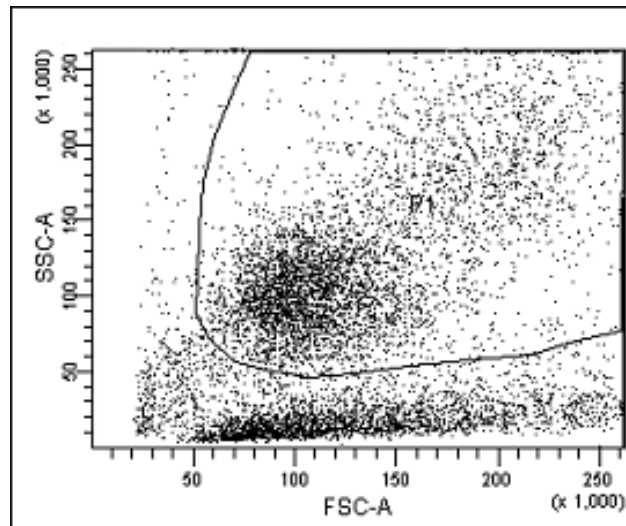


Figure 10: Forward scatter/side scatter (FSC/SSC) plot showing the cellular distribution in the air pouch 12 h after induction of inflammation through 1% Carrageenan injection (one representative C57BL/6 animal). Each dot represents one cellular event. FSC is proportional to cell size, SSC to cell granularity. Due to the early stage of inflammation a prominent population of neutrophils appears in the middle of the diagram. The gate P1 was set to exclude both cellular debris and Carrageenan particles.

In Fig. 11 a typical histogram of Ly6GC-stained cells is depicted, which shows a distinct, high-fluorescent peak representing the neutrophil population in the air pouch. In contrast, the unstained cells did not exhibit any specific fluorescence. For quantitative Flow Cytometry analysis only the high-fluorescent cells were regarded as neutrophils.

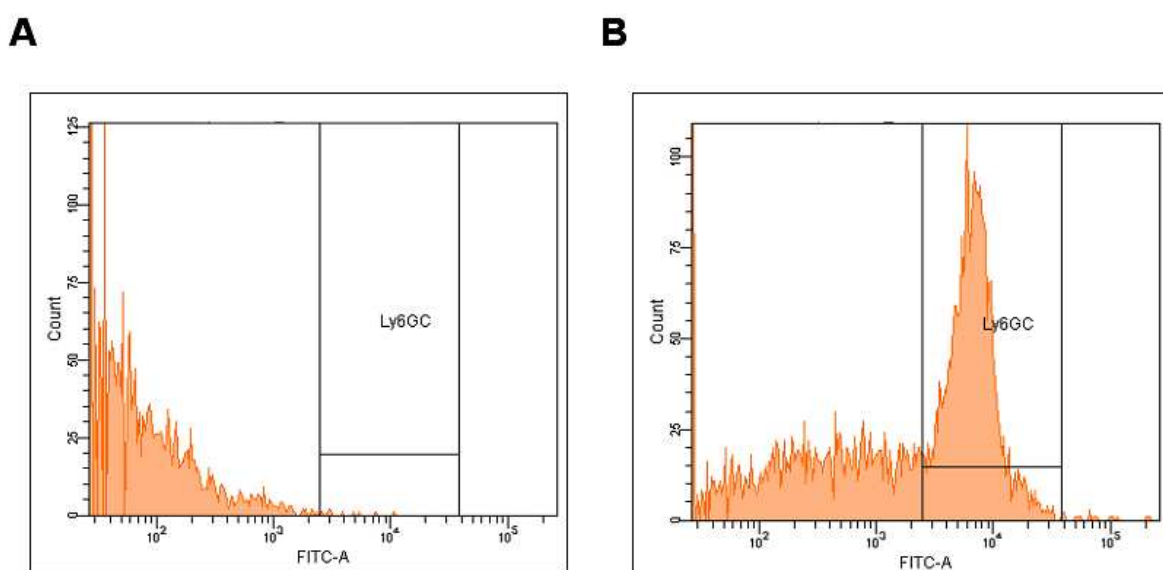


Figure 11: Flow Cytometry analysis (FITC-Channel) of neutrophils obtained from the pouch exudate of a representative C57BL/6 mouse, 12 h after Carrageenan injection. (A) Unstained cells. (B) Ly6GC-stained cells.

Since F4/80-staining did not yield such a clear and distinct peak, the proportion of macrophages was calculated from the difference in the fluorescence signal by comparing stained and unstained cells (Fig. 12).

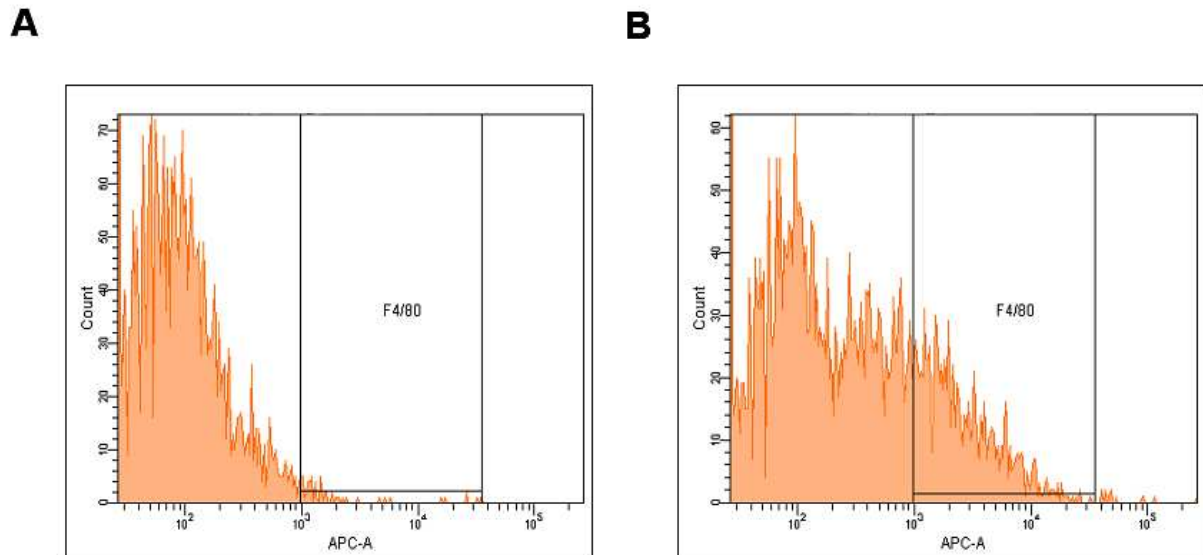


Figure 12: Flow Cytometry analysis (APC-Channel) of macrophages obtained from the pouch exudate of a representative C57BL/6 mouse, 12 h after Carrageenan injection. (A) Unstained cells. (B) F4/80-stained cells.

2.2.16 Intraperitoneal injection

400 μ l sterile PBS containing either purified anti-Ly6GC (neutrophil depletion) or recombinant Hyper-IL-6 (acute phase induction) were injected into the peritoneal cavity of an ether anesthetized mouse. To induce an acute phase response mice were treated intraperitoneally with 0.5 μ g Hyper-IL-6. 4 h after cytokine injection animals were sacrificed and the liver was removed. To deplete the neutrophils in the air pouch 100 μ g of purified anti-Ly6GC mAb were injected i.p. 18 h before Carrageenan challenge. Solutions were prepared and loaded under a sterile fume hood. Intraperitoneal injection was performed using a 1ml syringe in combination with a 27 gauge needle.

2.2.17 Statistical analysis

Data are expressed as mean values \pm SD; 4-7 mice were used per experimental group. Statistical analysis were 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 (**).

2.2.18 Animal treatment

Procedures involving animals and their care were conducted in conformity with national and international laws according to home office approved project license 66-6/06 from 9/15/2006. Mice were maintained in a 12 h light-dark cycle under standard conditions and were 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 $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $60\% \pm 5\%$ humidity in individually ventilated cages (IVC).

2.2.19 DNA microinjection

Microinjection of DNA into the pronucleus of a fertilized mouse egg is the method most extensively used in the production of transgenic mice. At best, the injected DNA integrates into the chromosomal DNA at the one-cell stage of the zygote and the transgenic mouse expresses the injected DNA in every cell including germ cells. For microinjection the vector containing the transgene DNA was cleaved with the appropriate restriction enzyme and the restriction fragment comprising the transgene was separated on a 1% agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen). Transgene DNA was dissolved in 5 mM Tris-HCl (pH 7.4), 0.1 mM EDTA and afterwards filtered to remove particles (0.22 μm). The microinjection was carried out at the Biotechnologielabor (BTL) of the University of Heidelberg.

2.2.20 Animal breeding

The transgene DNA was injected into B6D2F1 donor embryos. B6D2F1 is a hybrid strain created by a cross of a C57BL/6 (B6) female and a DBA/2 (D2) male mouse. B6D2F1 mice are heterozygous for B6 and D2 alleles at all loci in their genome. B6D2F1 mice are particularly suitable for DNA microinjection because of the increased fecundity, litter size, body weight, resistance to disease and overall life span compared to the inbred strains used to create it. After microinjection, viable zygotes were implanted into the oviducts of pseudopregnant foster mothers and the offspring was analyzed. Integration of the transgene was detected both by genomic Southern blotting and PCR using DNA isolated from tail biopsies of 3 to 4 week old mice. Founder animals were intercrossed with C57BL/6 mice to establish different transgenic lines. Once a transgenic line was established, heterozygous animals were crossed with each other to produce homozygous mice. This implicates that the homozygous transgenic animals used in this study were on a mixed genetic background. Therefore wild-type offspring of heterozygotes were used to establish a colony of genetically matched littermates, which were used as control animals throughout this work.

Since homozygous animals contain twice as much transgene DNA as heterozygous animals, homozygous mice can be discriminated from heterozygous by Southern blotting with the homozygous mice yielding twofold higher signal intensity compared to heterozygous animals. Moreover, homozygosity was verified through backcrossing with C57BL/6 wildtype animals (the complete offspring of a homozygous mouse mated with a wildtype animal should be heterozygous).

3 Results

3.1 The “first generation” of sgp130Fc-transgenic mice

3.1.1 Cloning of the transgene expression vector

The vector pTZ-pEPCK- β glob.intron was successfully used before to generate sIL-6R transgenic mice by pronuclear injection of DNA (89). sIL-6R transgenic mice have been shown to express the transgenic protein in large amounts (4-8 μ g/ml) in the serum (91). Therefore the plasmid pTZ-pEPCK- β glob.intron was used to generate a construct suitable for high expression of sgp130Fc in mice. The vector was opened with XbaI and a XbaI restriction fragment comprising the entire cDNA of human sgp130Fc, derived from the vector p409-sgp130Fc, was inserted.

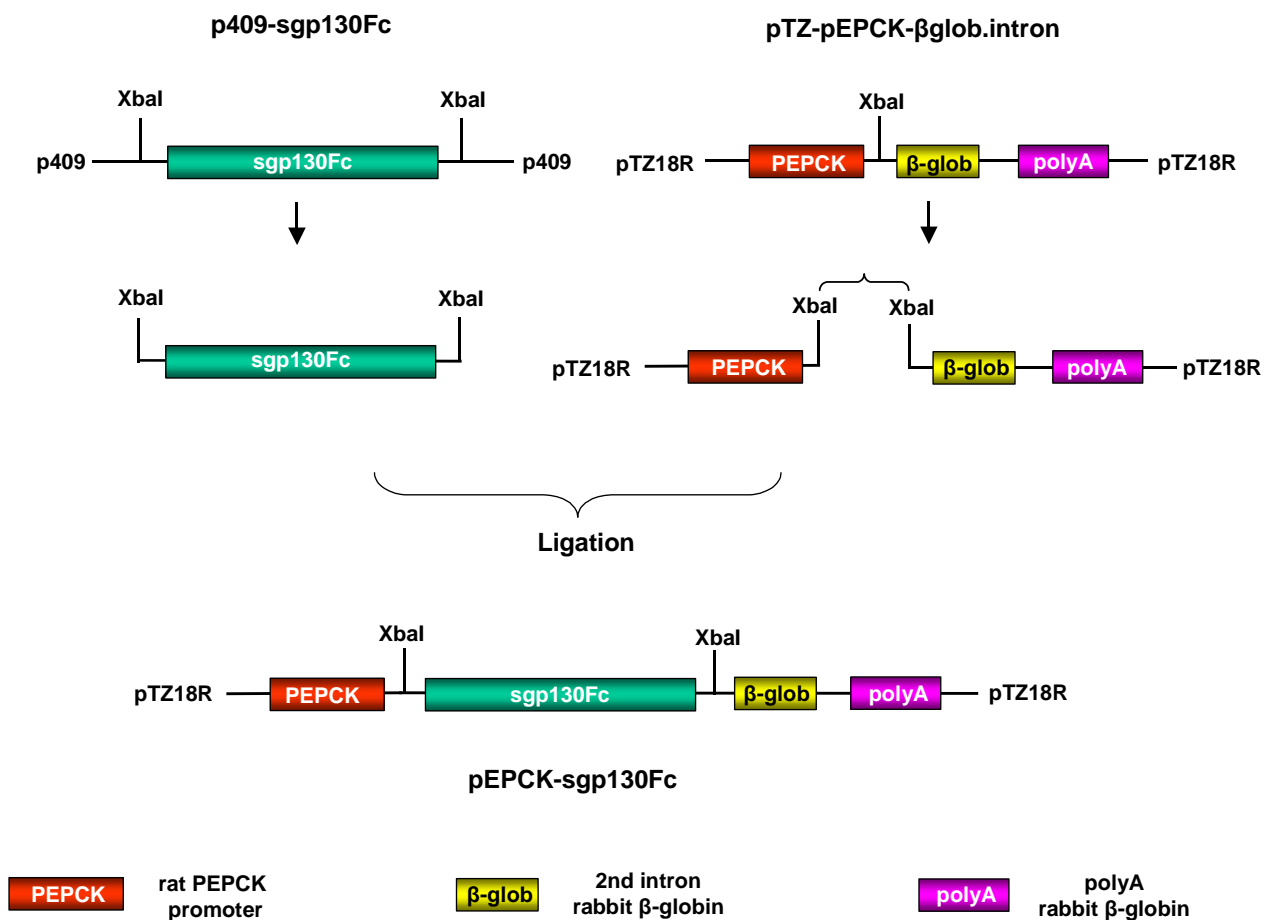


Figure 13: Cloning scheme for the vector pEPCK-sgp130Fc used for DNA microinjection.

15) the founder mice differ with respect to the signal intensity indicating that different copy numbers of the transgene have integrated into the genome.

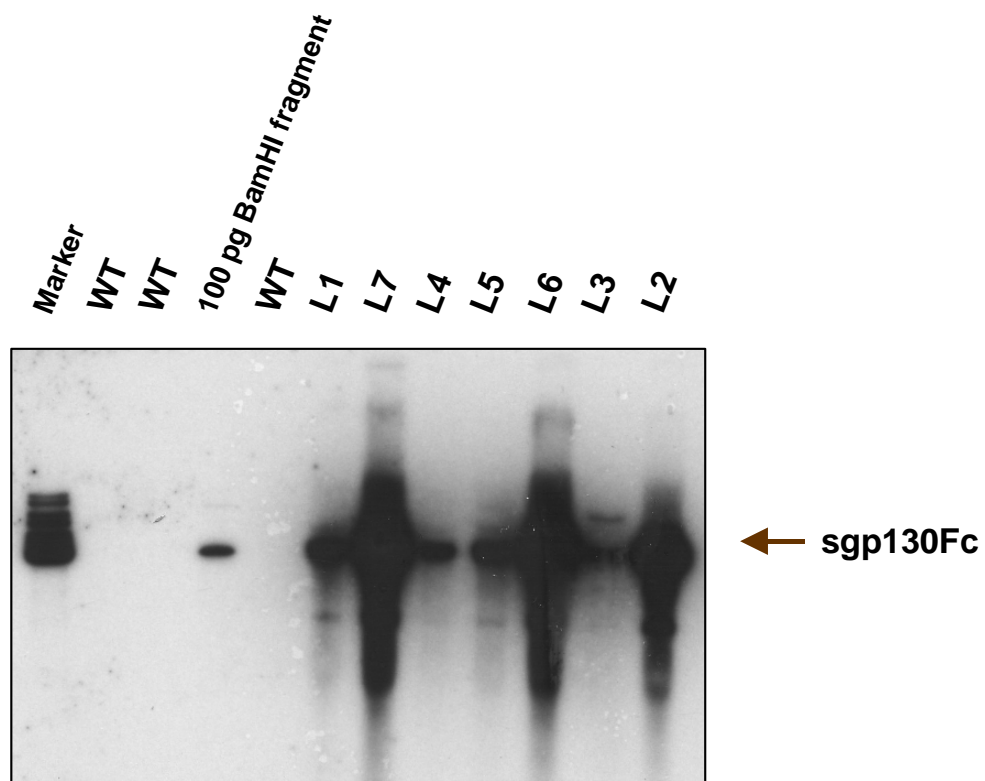


Figure 15: Southern analysis showing the seven transgenic founder animals (L1-L7) resulting from DNA microinjection. 10 μ g of genomic DNA was digested with NcoI and hybridized with a radioactive labeled probe consisting of the entire sgp130Fc cDNA. The absence of signals in the screened wildtype animals (WT) shows the high specificity of the employed DNA probe. 100 μ g BamHI restriction fragment of the vector pEPCK-sgp130Fc used for microinjection was included as a positive control.

Each founder animal was crossed with a C57BL/6 wildtype mouse to establish different transgenic lines, which were designated with L1-L7. To exclude the possibility of multiple integration sites, Southern analysis was also performed on the F1 litters to determine the segregation pattern of the transgene.

The lines L2, L5 and L7 were discarded during breeding due to the low transgenic protein levels detected in the serum or to bad breeding performance. The lines L1, L3, L4 and L6 were bred to homozygosity by mating two heterozygous animals of each transgenic line (in Fig. 16, by way of example, a breeding scheme for line L3 is outlined).

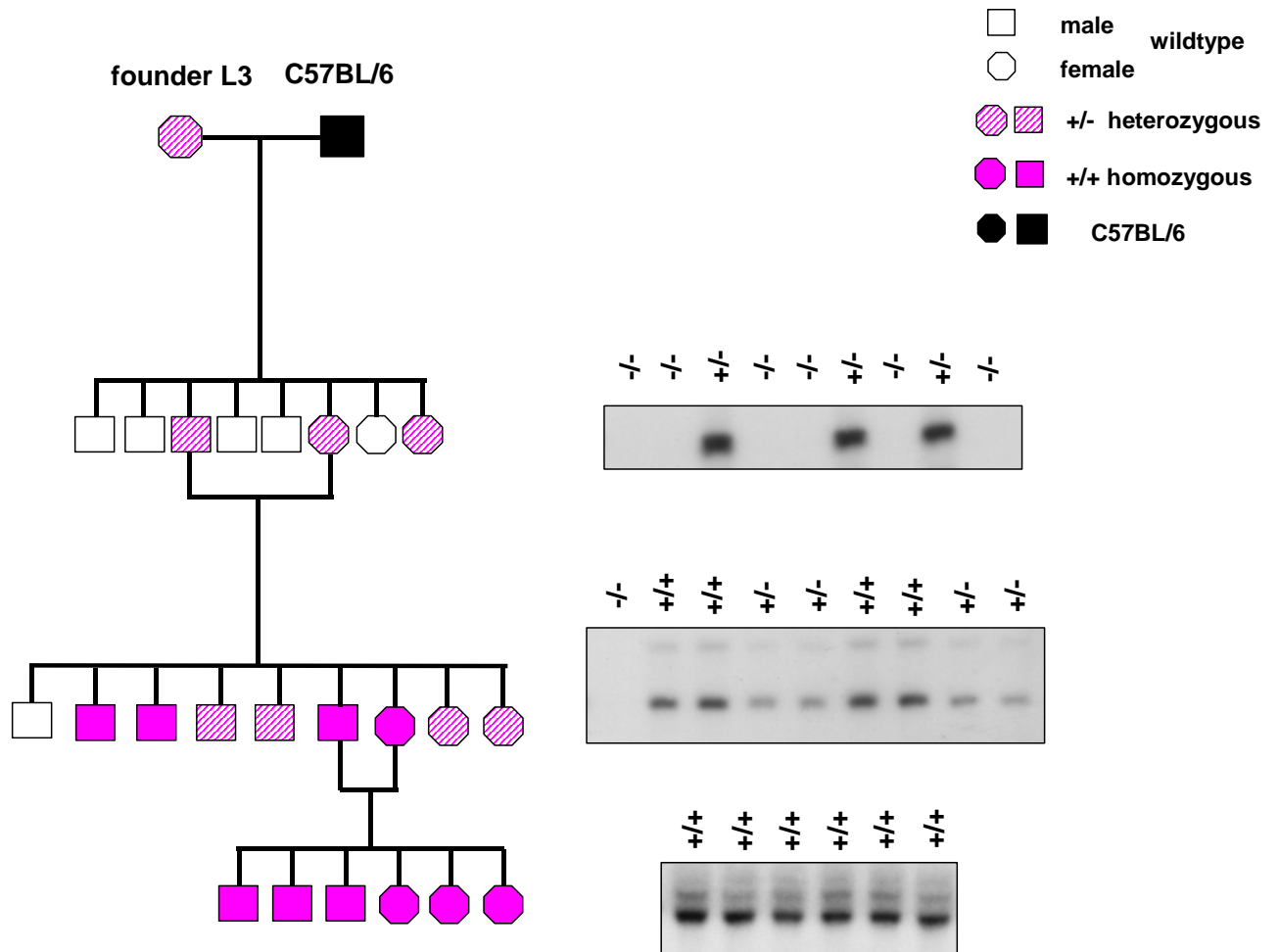


Figure 16: Breeding to homozygosity, as exemplified by line L3. The founder animal is intercrossed with a C57BL/6 wildtype mouse and two heterozygous littermates were bred to produce homozygous mice. Homozygous animals were mated to obtain exclusively homozygous offspring. In addition, the homozygous parents were crossed with C57BL/6 wildtype animals to test for true homozygosity. The breeding scheme is depicted on the left side with the corresponding Southern blots on the right.

3.1.3 Serum expression of transgenic sgp130Fc

Sgp130Fc is expressed under the transcriptional control of the PEPCK promoter, which is mainly active in liver and kidney. Since the liver exhibits a high synthetic capacity, transgenic proteins driven by the PEPCK promoter have been shown to be secreted at high amounts into the circulation (89). Therefore the serum levels of sgp130Fc were examined in homozygous animals. Sgp130Fc was precipitated from mouse serum with Protein A sepharose (binds the

Fc part of sgp130Fc) and detected by immunoblotting with a human sgp130-specific antibody.

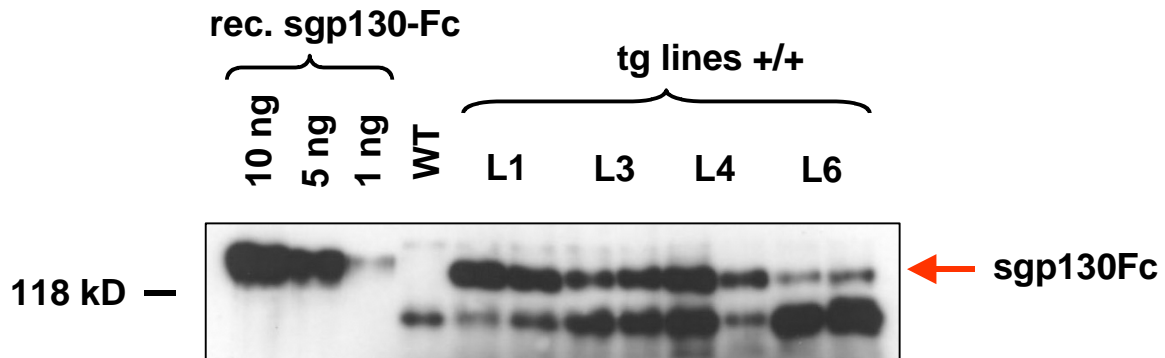


Figure 17: Detection of transgenic sgp130Fc in the serum of homozygous animals of the lines L1, L3, L4 and L6. Two mice per transgenic line were shown. As a positive control different amounts of recombinant sgp130Fc were included. In wildtype (WT) serum no sgp130Fc was found. The smaller band in the serum samples represents unspecific binding of the antibody, since it appears in the wildtype animal as well.

The highest concentration of sgp130Fc was observed in the serum of the lines L1 and L4, whereas line L3 showed a moderate, and line L6 the lowest expression of the transgene (Fig. 17). In order to quantify the exact serum levels of the transgenic protein, a commercial sgp130 sandwich ELISA (R&D) was employed. The sgp130Fc concentrations were in the high nanogram range. Consistent with the Western blot data, the highest transgene levels were measured in the lines L1 (0.39 μg sgp130Fc/ml serum) and L4 (0.55 $\mu\text{g}/\text{ml}$). Line 6 showed the weakest expression with 60 ng/ml serum (Fig. 18).

Remarkably, there was no obvious correlation between the copy number and the expression level of the transgene. Animals belonging to line L6 showed the lowest sgp130Fc serum levels. On the contrary, this line exhibited the strongest signal seen in the Southern analysis indicating a high copy number of the transgene. Moreover, the lines L1 and L4, which had the highest serum concentrations of sgp130Fc, only showed moderate signals in the Southern blot.

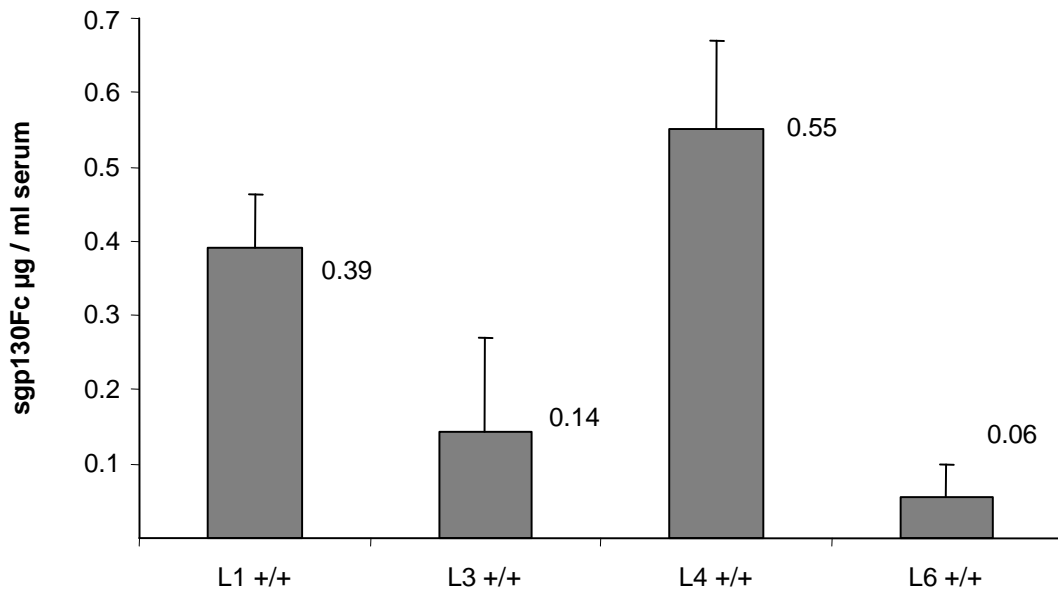


Figure 18: Serum levels of sgp130Fc in homozygous animals of the lines L1, L3, L4 and L6 as measured by sgp130 sandwich ELISA. Four animals per line were analyzed. Data represent mean values \pm SD.

The PEPCK promoter can be activated via dietary changes. During periods of fasting, starvation or intense exercise the body produces glucose from non-sugar carbon substrates like pyruvate, lactate, glycerol and amino acids (gluconeogenesis). For this reason it was possible to additionally increase the expression of sgp130Fc by feeding the mice a protein rich/carbohydrate low diet. Supplying the mice for two weeks with special food containing low amounts of carbohydrates resulted in a strong increase in protein expression (Fig. 19).

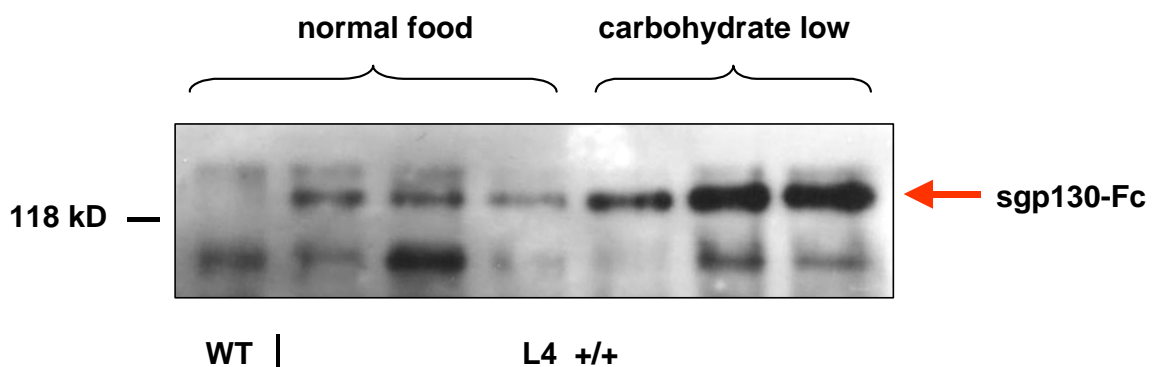


Figure 19: Comparison of normal and carbohydrate low diet (three mice per group). When homozygous mice of line L4 were fed for two weeks a special carbohydrate low diet, sgp130Fc serum concentration considerably increased. Sgp130Fc was precipitated with Protein A sepharose and detected by immunoblotting. WT wildtype animal.

3.1.4 Induction of the acute phase response via intraperitoneal injection of Hyper-IL-6

One of the most prominent features of IL-6 is its strong ability to stimulate the hepatic acute phase reaction in response to infection, physical trauma or malignancy (38). The acute phase response is characterized by changes in the plasma concentration of various so-called acute phase proteins (APPs), which are predominantly produced by the liver. Serum amyloid A (SAA) is a major acute phase protein in mice, which is induced up to a 1,000-fold under acute inflammatory conditions following exposure to bacterial lipopolysaccharides (125). Hyper-IL-6 is a strong inducer of the acute phase reaction as well and it turned out that the acute phase response, when compared to IL-6, is markedly increased and lasts significantly longer (88). In this experiment wildtype and homozygous *sgp130Fc* transgenic mice of line L4, which displayed the highest expression of *sgp130Fc* protein among the four transgenic lines, were injected intraperitoneally with 400 ng Hyper-IL-6. After four hours mice were sacrificed and the liver was removed. Total liver RNA was isolated and mRNA expression of the major acute phase protein SAA2 was monitored by Northern blotting (Fig. 20A).

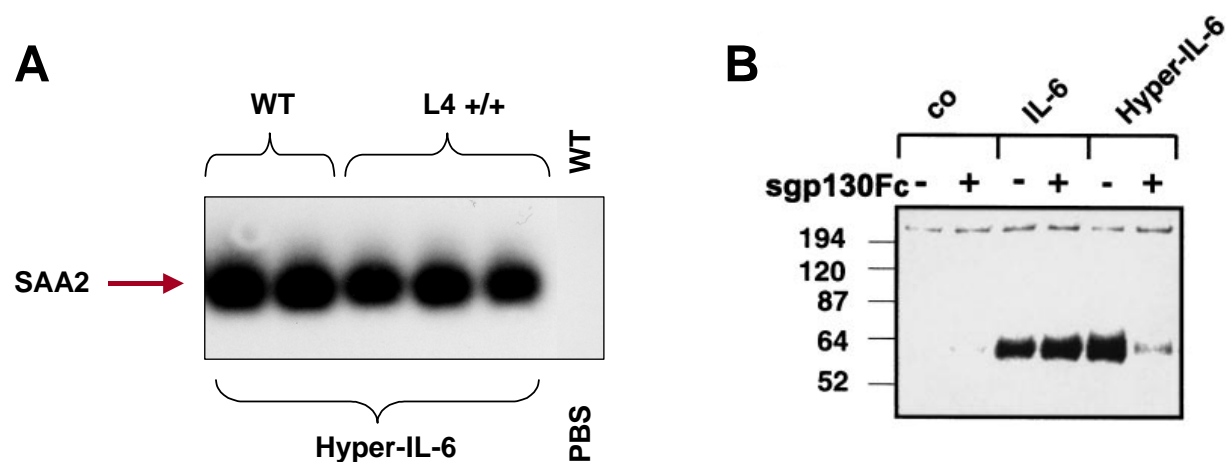


Figure 20: (A) Northern blot showing the liver expression of the APP serum amyloid A (SAA2) in two wildtype and three homozygous *sgp130Fc* transgenic mice of line L4 4 h after i.p. injection of 400 ng Hyper-IL-6. Transgenic mice were fed a carbohydrate low diet for two weeks. A wildtype animal (WT) injected with the same volume PBS served as a negative control. 5 μ g total liver RNA were separated on a 1% formaldehyde containing agarose gel and probed with a 32 P-labeled PCR fragment of the mouse SAA2 gene. (B) Sgp130Fc selectively inhibited the acute phase response induced by Hyper-IL-6. HepG2 cells were stimulated with 100 ng/ml of IL-6 or Hyper-IL-6 for 18 h in the presence or absence of 1 mg/ml *sgp130Fc*. Unstimulated cells treated with or without *sgp130Fc* were used as control (co). Secretion of the APP α 1-antichymotrypsin was analyzed by immunoblotting. Data taken from (55)

It was previously shown *in vitro* that sgp130Fc is able to selectively inhibit the acute phase response elicited by Hyper-IL-6, whereas the acute phase induced by IL-6 remained unaffected (Fig. 20B) (55).

Injection of Hyper-IL-6 into wildtype mice resulted in a strong induction of the acute phase protein SAA2. When sgp130Fc transgenic animals of line L4 were challenged with the same amount of Hyper-IL-6, no significant difference in terms of SAA expression was observed compared to wildtype mice. Intraperitoneal injection of the carrier solution (PBS) caused no response indicating that the injection *per se* did not lead to a stimulation of the acute phase reaction. Taken together, these results suggest that the amounts of sgp130Fc present in the circulation of homozygous animals of line L4 were not sufficient to block the acute phase response induced by Hyper-IL-6.

3.2 The “second generation” of sgp130Fc transgenic mice

A molar excess of sgp130Fc is needed for an effective inhibition of IL-6-transsignaling, as sgp130Fc has to compete with the membrane-bound gp130 for the IL-6/sIL-6R complex (55). Although the expression of sgp130Fc could further be enhanced by feeding the mice a carbohydrate low diet, the amounts of circulating sgp130Fc in the transgenic mice were nevertheless too low to inhibit the Hyper-IL-6-induced acute phase response. In particular, when considering microenvironments as the knee joint, where high levels of sIL-6R (24.7 ng/ml) have been detected in rheumatoid arthritis patients (23), it is important to have sufficient serum levels of sgp130Fc to effectively block sIL-6R mediated signaling in such closed compartments within the mouse. In order to achieve higher serum concentrations of sgp130Fc, transgenic mice were generated, which express sgp130Fc from a codon-optimized cDNA.

3.2.1 Codon-optimization of the sgp130Fc cDNA

Codon-optimization was performed by the biotech company GENEART (Regensburg). The codon usage of sgp130Fc was adapted to the codon bias of mammalian genes, i.e. only the most frequently occurring codons in mammalian genomes were used. By increasing the

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translation efficiency – without altering the amino acid sequence - the optimized gene should allow high and stable expression rates in human as well as in rodent cells.

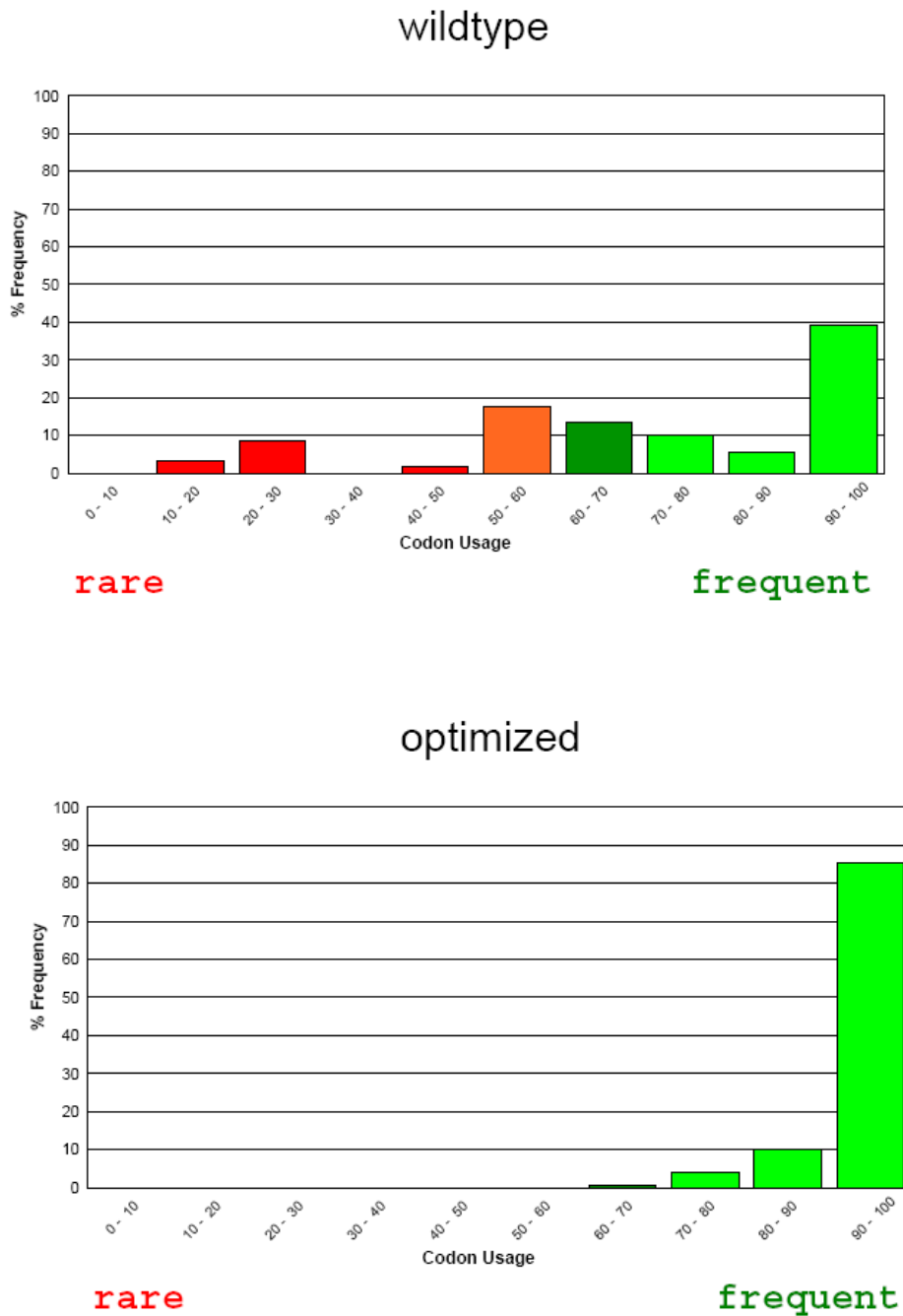


Figure 21: Codon usage of the optimized gene (sgp130Fc) compared to the wildtype sequence: the most frequently used codon was set to 100 and the remaining were scaled accordingly (“relative adaptiveness”). The histograms represent the overall codon frequency within the coding region; e.g. red bars indicate the frequency of extremely rare codons.

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In addition, regions of very high (>80%) or very low (<30%) GC content were avoided where possible, thereby increasing mRNA half-life.

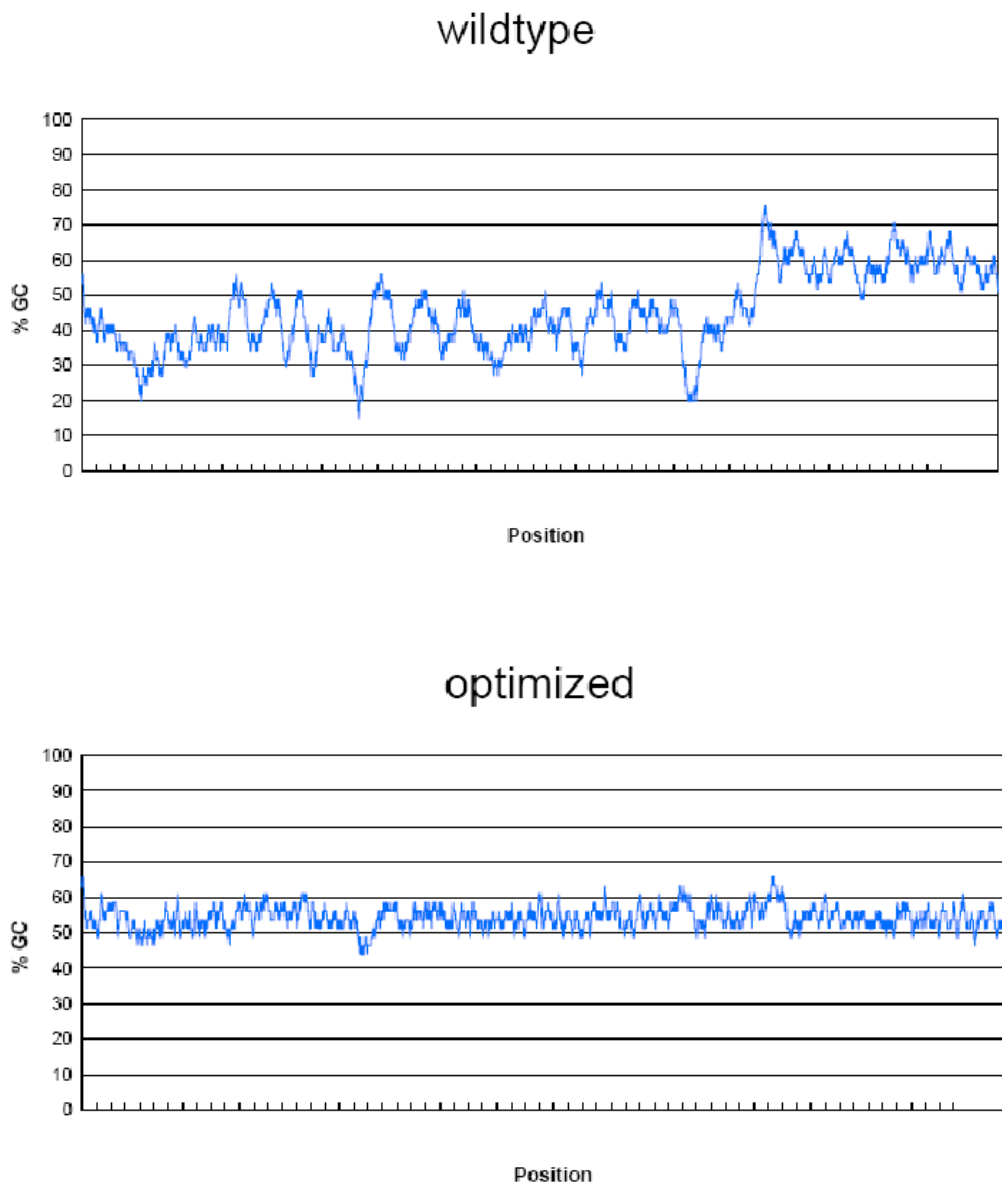


Figure 22: GC content of the optimized gene (sgp130Fc) compared to the wildtype sequence: The plot represents the GC content at the indicated sequence position

During the optimization process, the following cis-acting sequence motifs, which may negatively influence the expression, were eliminated:

- internal TATA-boxes, chi-sites (recombinational hotspots) and ribosomal entry sites
- AT-rich or GC-rich sequence stretches
- repeat sequences and RNA secondary structures
- cryptic splice donor and acceptor sites, branch points
- reverse-complementary sequence identities longer than 20 nucleotides (to avoid RNA interference)

As a result of the cDNA optimization ~ 23% of the wildtype nucleotides were exchanged. Moreover, for an efficient translational termination a second stop codon was added and a Kozak-consensus sequence (short) was introduced upstream of the starting ATG in order to improve translational initiation.

Additional information is available on the homepage of GENEART (www.geneart.com).

3.2.2 Cloning of the transgene expression vector, part II

Beside the codon-optimization of the sgp130Fc cDNA, we chose an additional method to enhance the expression of the transgene. It is known that genomic constructs (containing introns and exons) were expressed more efficiently in transgenic mice than constructs lacking introns (10). Further improvement of expression efficiency could be achieved when heterologous introns were inserted between promoter and the transgene, and not 3' of the coding region (86). Therefore, the codon-optimized cDNA of sgp130Fc was cloned between the 2nd intron and the polyA addition signal of the rabbit β -globin gene. The vector pTZ-pEPCK- β glob.intron was opened with XhoI and then ligated with a XhoI restriction fragment comprising the entire optimized sgp130Fc cDNA, which was derived from the vector p409-sgp130Fc/opt (Fig. 23A). Moreover, optimized sgp130Fc was inserted between the rat PEPCCK promoter and the 2nd β -globin intron. For this purpose the vector pTZ-pEPCK- β glob.intron was digested with XbaI, made blunt-ended with T4 DNA Polymerase and then ligated with a blunt PmeI restriction fragment, that was also derived from the vector p409-sgp130Fc/opt and contained the complete sgp130Fc/opt cDNA (Fig. 23B). Thus, in the two resulting constructs the optimized sgp130Fc cDNA was located in front of as well as behind the 2nd β -globin intron.

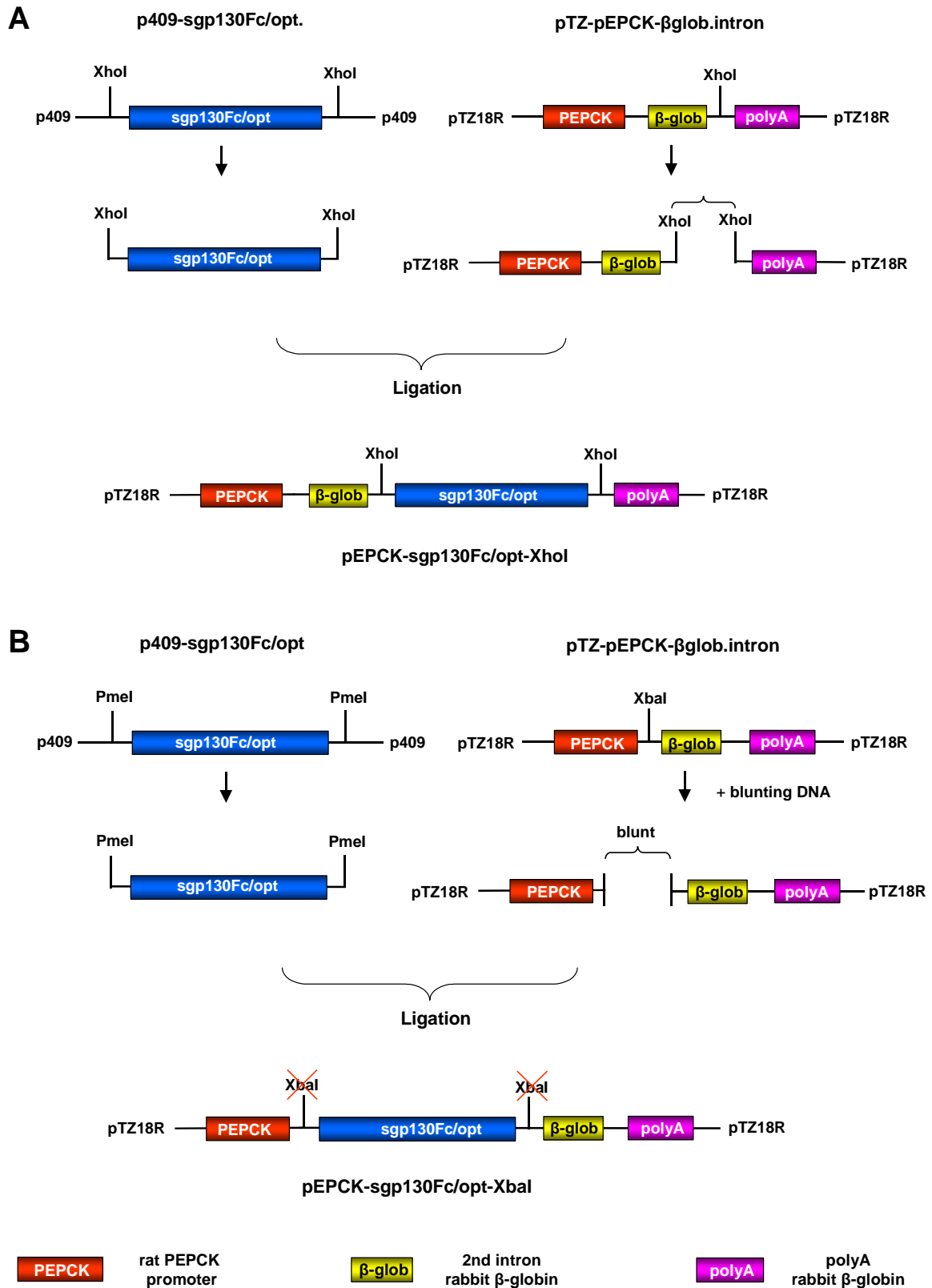


Figure 23: Cloning scheme for the vectors pEPCK-sgp130Fc/opt-XhoI (A, optimized sgp130Fc cDNA located behind the intron) and pEPCK-sgp130Fc/opt-XbaI (B, optimized sgp130Fc cDNA located in front of the intron). The XbaI restriction sites in the resulting pEPCK-sgp130Fc/opt-XbaI vector are destroyed due to blunting of the DNA after digestion.

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Finally, for a proper comparison of the effect resulting from codon-optimization with the position effect of the intron the wildtype human *sgp130Fc* cDNA was cloned behind the 2nd β -globin intron. Therefore, the transgene expression vector pTZ-pEPCK- β glob.intron was cleaved with *Xho*I and subsequently blunted with T4 DNA Polymerase. A blunt *Pme*I restriction fragment comprising the entire wildtype *sgp130Fc* cDNA, originated from the vector p409-*sgp130Fc*, was then ligated into the opened vector (Fig. 24).

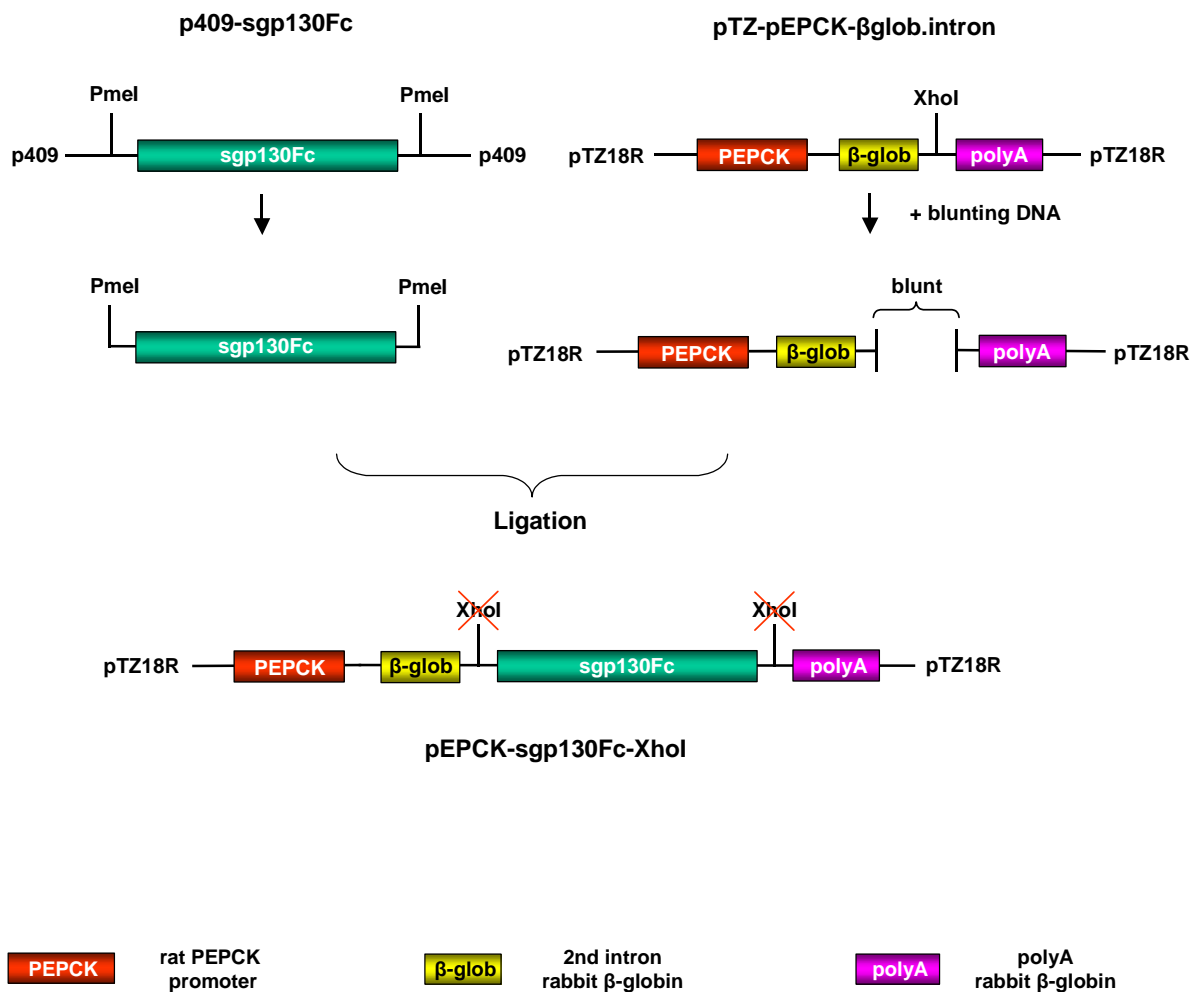


Figure 24: Cloning scheme for the vector pEPCK-sgp130Fc-XhoI (wildtype *sgp130Fc* cDNA, located behind the intron). The *Xho*I restriction sites in the resulting pEPCK-sgp130Fc-XhoI vector are destroyed due to blunting of the DNA after digestion.

3.2.3 Comparative expression of optimized sgp130Fc *in vitro*

The original expression vector pEPCK-sgp130Fc, which was previously used in this work to generate sgp130Fc transgenic mice, and the three derivatives pEPCK-sgp130Fc-XhoI, pEPCK-sgp130Fc/opt-XhoI and pEPCK-sgp130Fc/opt-XbaI were transiently expressed in the human hepatoma cell line HepG2. As expression of sgp130Fc in this vector is driven by the rat pEPCK promoter, which has been shown to be specific in liver and kidney, the liver cell line HepG2 is an ideal model system to monitor sgp130Fc expression *in vitro*. A simplified schematic representation of the four different sgp130Fc transgene expression cassettes is given in Fig. 25A.

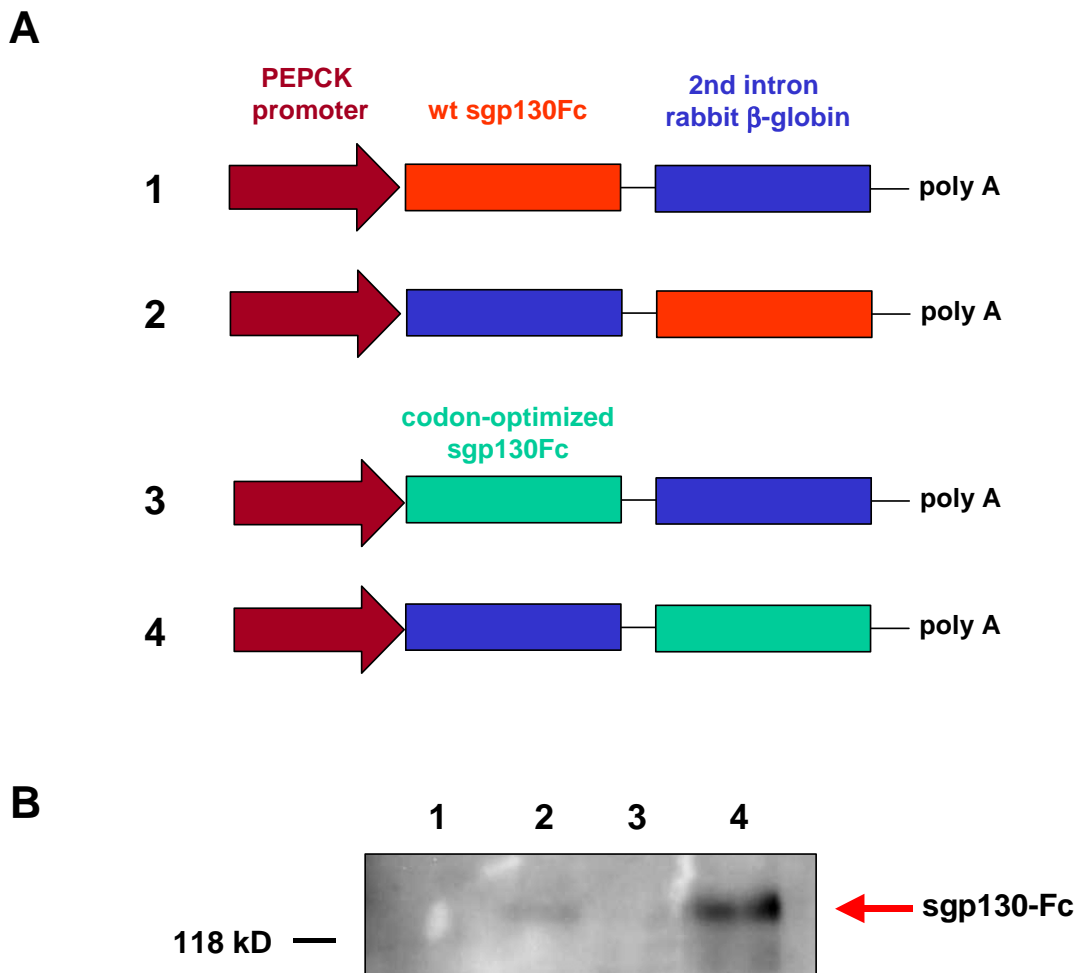


Figure 25: (A) Different transgene expression cassettes. The constructs differ in 1. the position of the intron (in front of/behind the transgene) and 2. in the optimization status (wildtype/optimized cDNA). 1. pEPCK-sgp130Fc, 2. pEPCK-sgp130Fc-XhoI, 3. pEPCK-sgp130Fc/opt-XbaI, 4. pEPCK-sgp130Fc/opt-XhoI. (B) Different expression levels of the sgp130Fc constructs in transfected HepG2 cell supernatant. Sgp130Fc was precipitated from cell supernatant with Protein A sepharose and detected by immunoblotting

To analyze and compare the expression of the four different transgene expression constructs *in vitro*, sgp130Fc protein was precipitated from 10 ml transfected HepG2 cell supernatant with Protein A Sepharose and detected by Western blotting. As depicted in Fig. 25B, the strongest expression was observed when codon-optimized sgp130Fc was located behind the 2nd β -globin intron. A clearly weaker expression was found when wildtype sgp130Fc was cloned behind the intron. However, when the transgene was located in front of the 2nd β -globin intron, expression of sgp130Fc was hardly detectable in HepG2 supernatant.

Since the pEPCK-sgp130Fc/opt-XhoI expression vector led to the highest expression rates of sgp130Fc *in vitro*, this construct was used to create the „second generation” of sgp130Fc transgenic mice. In Fig. 26 a schematic diagram of the pEPCK-sgp130Fc/opt-XhoI expression cassette is depicted, which shows all relevant functional regions and restriction sites.

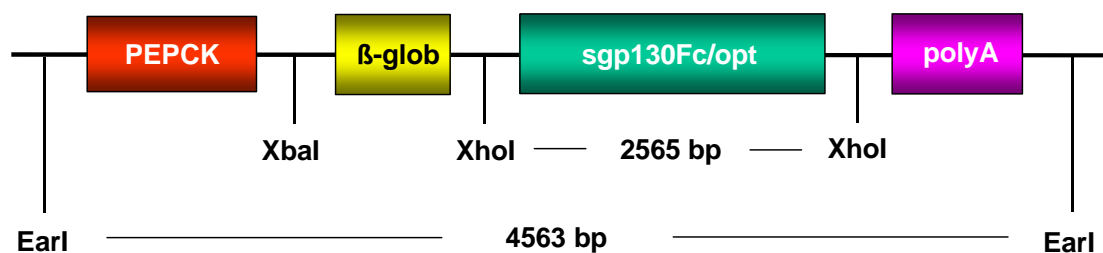


Figure 26: Schematic organization of the transgene expression cassette pEPCK-sgp130Fc/opt-XhoI.

3.2.4 Generation of sgp130Fc transgenic mice, part II

For the generation of codon-optimized sgp130Fc transgenic mice the EarI restriction fragment containing the full sgp130Fc/opt expression cassette was injected into the pronuclei of fertilized mouse eggs. From 128 pups, which arose from DNA microinjection, 14 were tested as transgene positive by Southern blotting and PCR (10.9% transgene transmission). Due to the high number of putative founder animals sgp130Fc serum expression was assessed and only the founders with the highest transgene expression were used for further breeding. Four different transgenic lines were established (as described in 3.1.2.), which were designated as opt1, opt2, opt3 and opt4. As seen in the Western blot in Fig. 27, the four transgenic lines apparently show different sgp130Fc serum levels with the highest sgp130Fc concentration

being observed in the serum of the founder animal of line opt2. The amounts of transgenic sgp130Fc in the serum of the remaining founders opt1, opt3 and opt4 were roughly the same, whereas sgp130Fc expression in line opt3 appeared to be slightly stronger than in the lines opt1 and opt4.

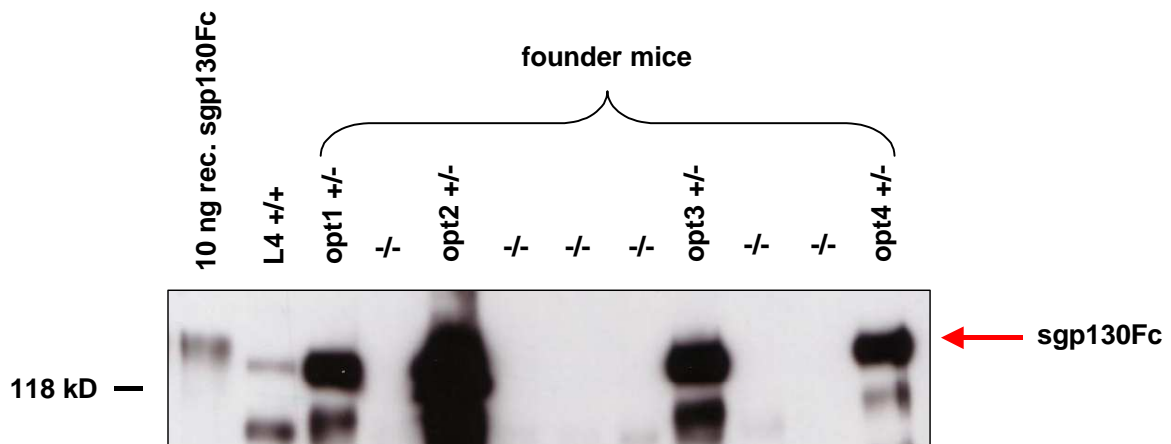


Figure 27: Different sgp130Fc levels in the serum of the founder animals of the lines opt1-4. Serum of a homozygous animal of the line L4 (first sgp130Fc transgenic mice) and 10 ng recombinant sgp130Fc were included as positive controls. Animals indicated with -/- were either wildtype animals or mice, which were tested positive for the transgene by PCR or Southern blot but failed to produce the transgenic protein. Sgp130Fc was precipitated from serum with Protein A sepharose and detected by immunoblotting.

The highest sgp130Fc expression among the four transgenic lines of the first generation of sgp130Fc transgenic mice was measured in line L4. The homozygous animal of line L4, which was included in the Western blot analysis as a positive control, only yielded a faint band indicating a relatively weak sgp130Fc expression compared to the lines opt1-4. It is also worth mentioning that in contrast to the L4 animal the founder animals of the lines opt1-4 were only heterozygous and therefore tended to produce rather lower amounts of the transgene. However, by comparing the sgp130Fc serum levels of the first sgp130Fc transgenic mice with the founder animals of the newly generated transgenic mice it clearly turned out that cDNA codon-optimization together with an altered position of the intron led to a potent amplification of transgenic sgp130Fc expression. The lines opt2 and opt3, which showed the highest sgp130Fc serum expression, were bred to homozygosity. In contrast, the transgenic lines opt1 and opt4 were discarded due to a bad breeding performance and to comparatively low sgp130Fc serum levels.

3.2.5 Serum expression of transgenic sgp130Fc

As mentioned above, sgp130Fc expression could be markedly increased in the newly-created codon-optimized sgp130Fc transgenic mice. The exact serum concentrations of sgp130Fc in the remaining lines opt2 and opt3 were quantified by sgp130 sandwich ELISA (R&D), as shown in Fig. 28.

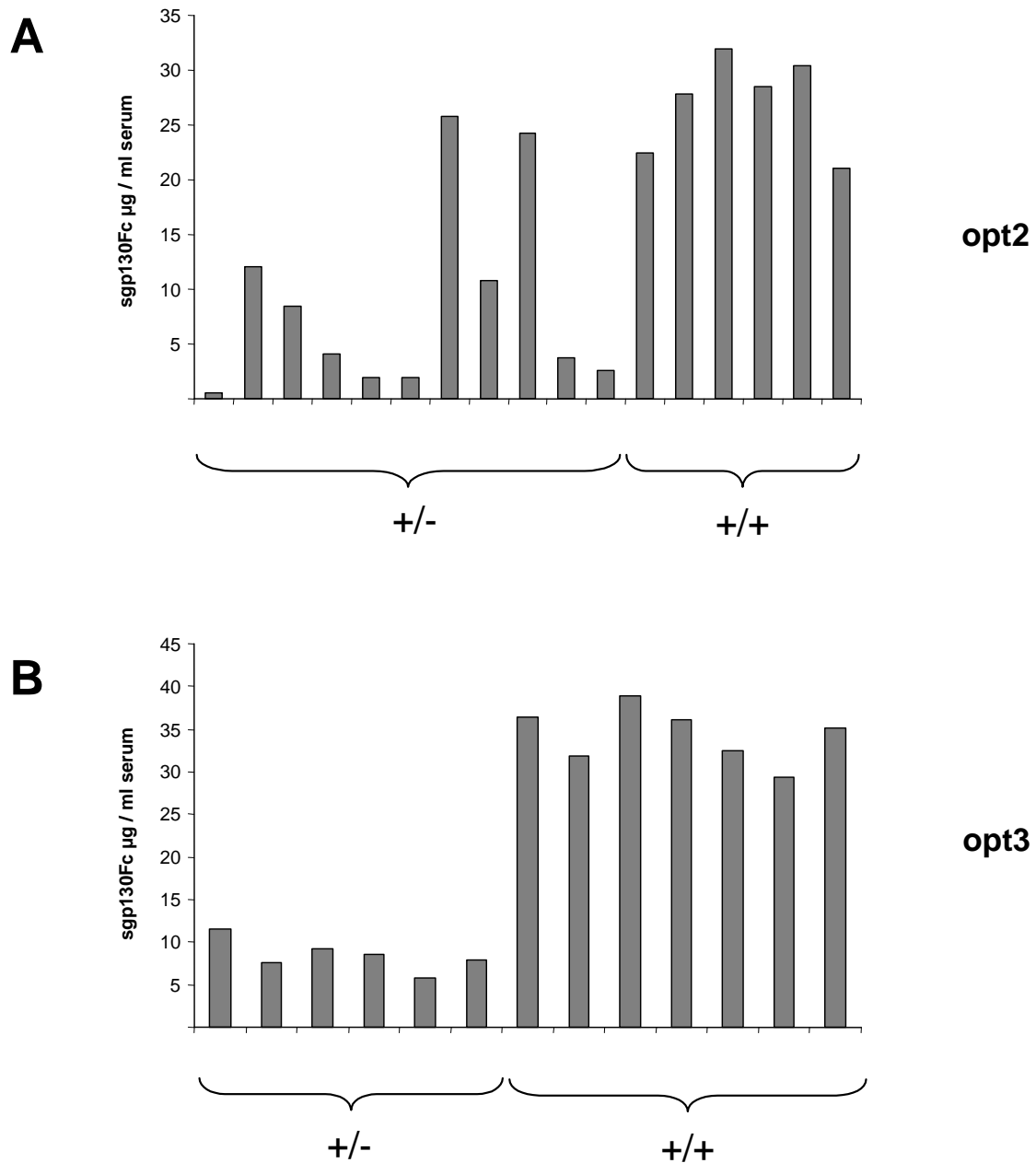


Figure 28: Varying sgp130Fc serum concentrations in heterozygous (+/-) and homozygous (+/+) animals of the lines opt2 (A) and opt3 (B) as measured by ELISA. Each bar represents one mouse.

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Taken together, for both lines the transgene serum levels of homozygous mice were significantly higher when compared to the heterozygous littermates. The sgp130Fc serum concentrations in the homozygous animals were in the 20-40 microgram range. The highest levels were measured in homozygous animals of line opt3 ($34.3 \pm 3.3 \mu\text{g/ml}$ serum), whereas sgp130Fc serum concentration in homozygous animals of line opt2 was only marginally below this value ($27 \pm 4.4 \mu\text{g/ml}$).

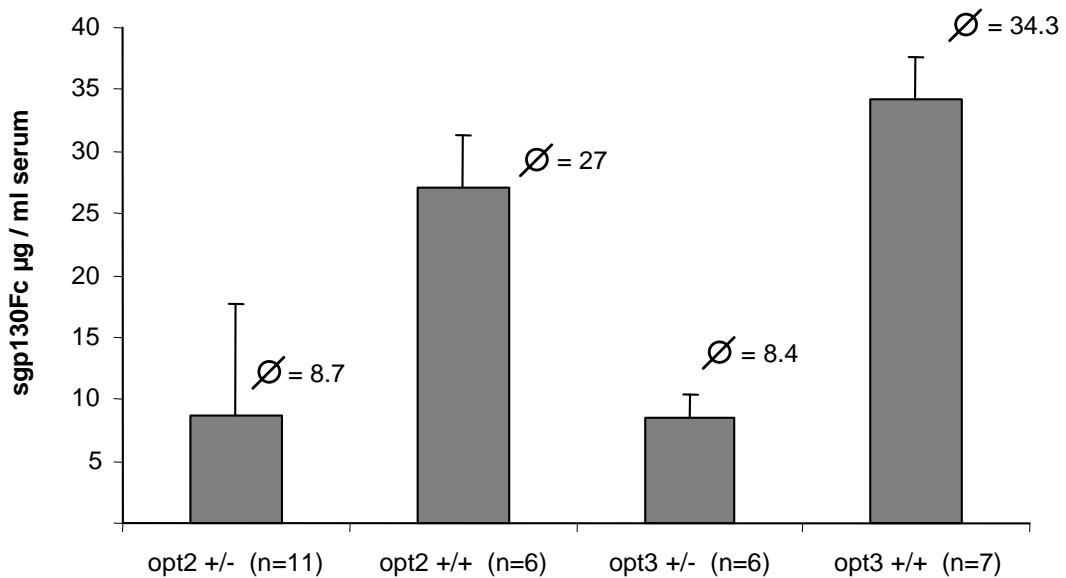


Figure 29: Mean sgp130Fc serum concentration in heterozygous (+/-) and homozygous (+/+) animals of the lines opt2 and opt3 as measured by ELISA. Data represent mean values \pm SD. n mice per group.

By breeding the transgenic mice to homozygosity sgp130Fc expression in animals of the line opt3 could be amplified by the factor 4 ($8.4 \rightarrow 34.3 \mu\text{g/ml}$). All heterozygous as well as all homozygous mice of the line opt3 showed similar sgp130Fc serum levels indicating a stable expression pattern of the transgene. In contrast, the situation observed in opt2 heterozygous mice was more complex, since sgp130Fc expression in the heterozygous animals was somewhat variable, albeit the mean sgp130Fc levels in heterozygous mice of both lines were in the same range. Some of the opt2 heterozygous mice reached the sgp130Fc concentrations obtained for homozygous animals, whereas other values were only in the high nanogram range. This inconsistent expression pattern obtained for heterozygous animals of line opt2 is reflected by the relatively large error bar shown in Fig. 29. Interestingly, the expression level

of sgp130Fc were similar in the different opt2 homozygous mice. When comparing homozygous mice of the “first” with homozygous animals of the “second generation”, a more than 50-fold increase of sgp130Fc protein in the serum was achieved.

3.2.6 Differential tissue expression of transgenic sgp130Fc

It has been reported that transgenic proteins under the control of the PEPCCK promoter were mainly expressed in liver and – to a lesser extent – in kidney (70). To examine the transgene expression profile in sgp130Fc transgenic mice, total RNA was isolated from liver, kidney, spleen, lung, brain, muscle, gut and thymus. Sgp130Fc mRNA expression was assessed by Northern blotting using a sgp130Fc-specific probe (Fig. 30).

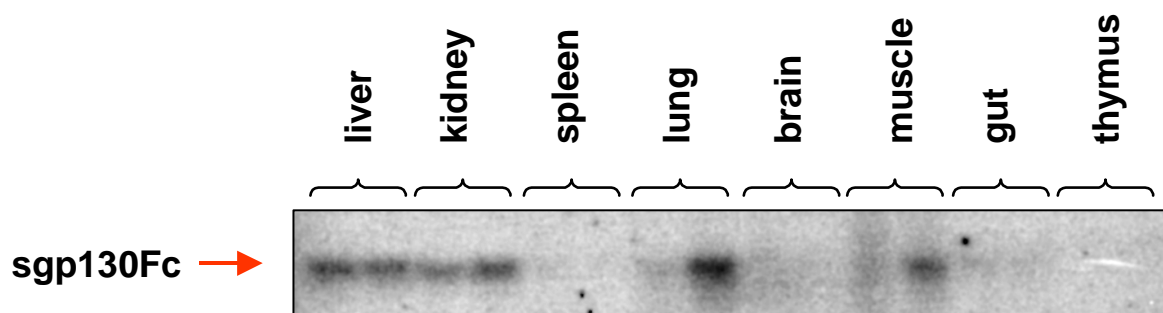


Figure 30: Northern blot showing sgp130Fc mRNA expression in different organs of two homozygous transgenic mice. 8 µg total liver RNA were separated on a 1% formaldehyde containing agarose gel and then hybridized with a ³²P-labeled XhoI restriction fragment comprising the complete sgp130Fc/opt cDNA.

Consistently, sgp130Fc expression was found mainly in liver and kidney. However, appreciable amounts of sgp130Fc mRNA could also be detected in lung and muscle. Little or no sgp130Fc expression was observed in spleen, brain, gut and thymus.

3.2.7 Transgenic sgp130Fc is functionally active

3.2.7.1 Transgenic sgp130Fc can precipitate Hyper-IL-6

Hyper-IL-6, which is a fusion protein consisting of IL-6 and sIL-6R (see introduction), is known to bind sgp130Fc in solution. To test if the sgp130Fc protein produced by transgenic mice was functionally active, serum of both wildtype and transgenic mice was mixed with recombinant Hyper-IL-6. Transgenic sgp130Fc was then precipitated from serum with Protein A sepharose and the co-precipitated Hyper-IL-6 was detected by immunoblotting (Fig. 31).

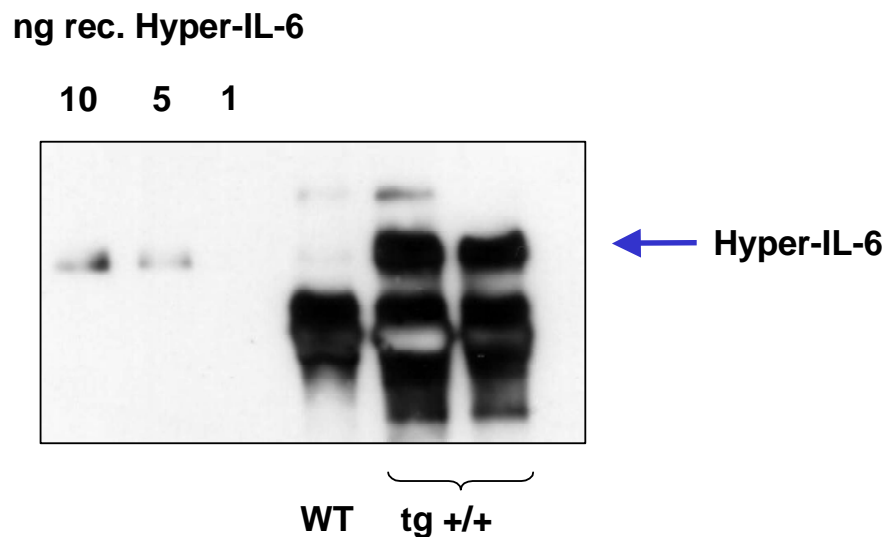


Figure 31: Hyper-IL-6 co-precipitates with the sgp130Fc, that is present in the serum of sgp130Fc transgenic mice. Hyper-IL-6 was detected by Western blotting against sIL-6R. Two homozygous animals of the line opt3 and one wildtype animal are shown. Three different concentrations of recombinant Hyper-IL-6 were included as positive controls.

Sgp130Fc protein, which is produced from sgp130Fc transgenic mice, is able to bind and precipitate recombinant Hyper-IL-6 indicating that the transgenic protein is functionally active. Incubation of wildtype serum, in which no sgp130Fc is present, with recombinant Hyper-IL-6 yielded no signal in the Western blot.

3.2.7.2 Induced acute phase response is suppressed in sgp130Fc transgenic animals

To test if the amounts of sgp130Fc produced from transgenic animals of the “second generation” were sufficient to inhibit murine IL-6-transsignaling, mice were again challenged with Hyper-IL-6. To this end wildtype and sgp130Fc transgenic mice were injected intraperitoneally with 500 ng recombinant Hyper-IL-6. After four hours mice were sacrificed and total liver RNA was isolated. Expression of the major acute phase protein SAA2 was assessed by Northern blotting (Fig. 32).

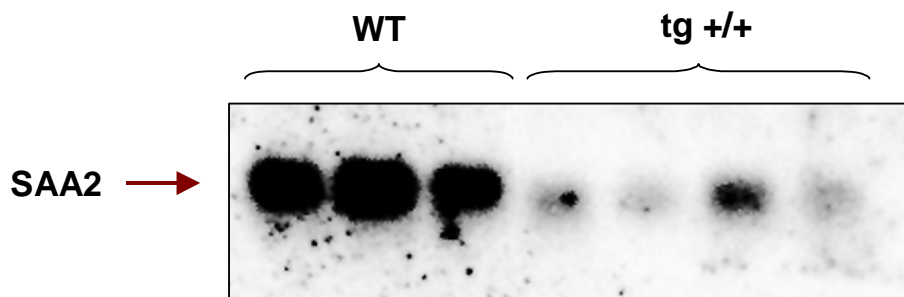


Figure 32: Northern blot showing the expression of the major acute phase protein SAA2 in the liver of three wildtype and four homozygous sgp130Fc transgenic mice 4 h after i.p. injection of 500 ng Hyper-IL-6. 5 μ g total liver RNA were separated on a 1% formaldehyde containing agarose gel and probed with a 32 P-labeled PCR fragment of the mouse SAA2 gene.

Strong SAA2 expression was observed in wildtype animals upon injection of 500 ng Hyper-IL-6. On the contrary, in sgp130Fc transgenic animals the mRNA expression of SAA2 was clearly diminished upon cytokine challenge indicating that in transgenic mice of the “second generation” the amounts of sgp130Fc protein were sufficient to inhibit an induced acute phase response. This is in contrast to the results obtained from transgenic mice of the “first generation”, which apparently did not express enough sgp130Fc protein to exhibit an efficient inhibition. In addition, this experiment clearly demonstrates that the transgenic sgp130Fc was functionally active.

3.3 Air pouch model of acute inflammation

Subcutaneous injection of sterile air under the dorsal skin of mice results in the formation of an air pouch with a lining morphologically and functionally similar to the synovium. The non-inflamed cavity is lined by a thin layer of fibroblasts and macrophage-like cells. The introduction of an irritant into the cavity induces a local inflammatory response that resembles that seen in the rheumatoid joint (29, 107). Carrageenan, which was used in this work as the inflammatory stimulus, is a sulphated polysaccharide obtained from the alga *Chondrus crispus*. It has been widely used experimentally for its ability to induce an acute inflammatory response (24).

Since several recent studies have implicated sIL-6R mediated signaling in the accumulation of leukocytes at sites of infection/inflammation (49, 96, 116), the murine air pouch model was employed to investigate the consequences of permanent inhibition of IL-6-transsignaling in sgp130Fc transgenic mice during an acute inflammatory episode.

3.3.1 IL-6 and sIL-6R levels in the inflamed air pouch

1% Carrageenan solution was administered into 6-day-old air pouches of 8-10 week old male C57BL/6 wildtype mice to induce an acute inflammatory response (Fig. 33). The same volume of sterile PBS was injected into the air pouches of other C57BL/6 animals as a negative control (non-inflamed air pouch).

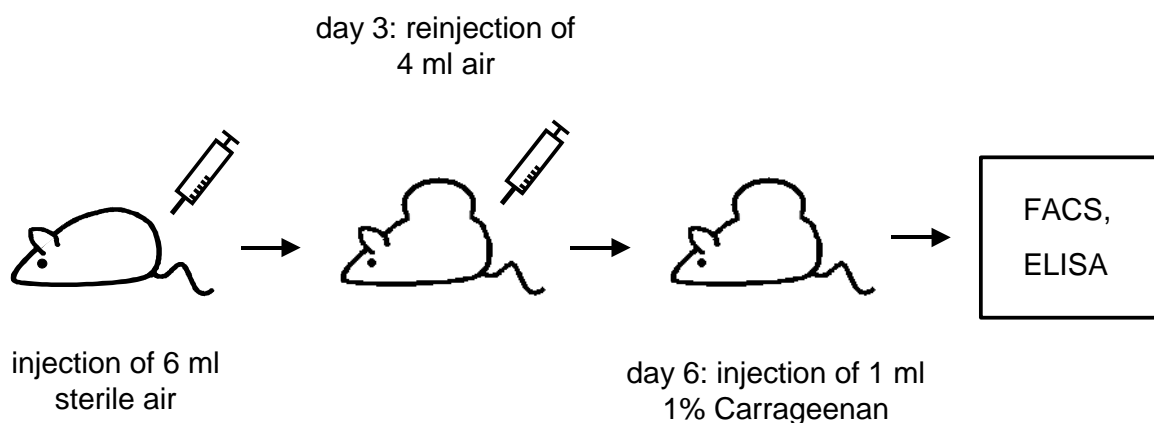


Figure 33: Schematic representation of the air pouch time schedule. At different time points after Carrageenan challenge the pouch was washed with 3 ml PBS and the exudate was analyzed by FACS (differential cell counts) or ELISA (cytokines/soluble cytokine receptors).

Levels of murine IL-6 and murine sIL-6R were assessed in the exudate fluid over a time period of three days using sandwich ELISA (Fig. 34).

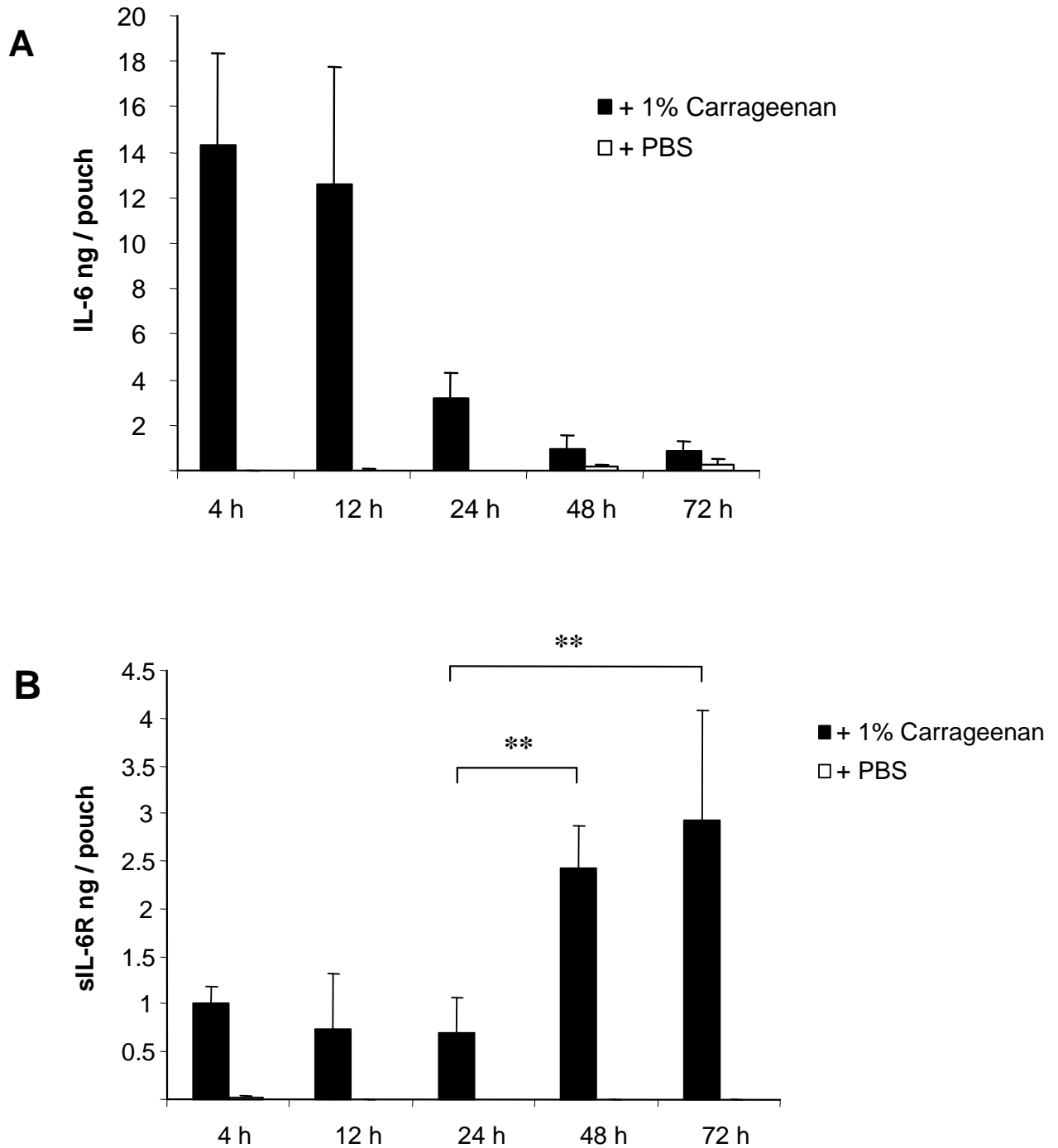


Figure 34: Levels of endogenous IL-6 (A) and sIL-6R (B) in inflamed (1% Carrageenan) and non-inflamed (PBS) air pouches at different time points postchallenge as measured by ELISA. 4-7 mice per time point. In non-inflamed air pouches IL-6 and sIL-6R were hardly detectable. Data are represented as mean values \pm SD (**= $p < 0.01$).

IL-6 levels peaked early (4 h) and declined rapidly to about 1000 pg/pouch (48 h and 72 h). On the contrary, sIL-6R levels plateaued at early time points (~ 1 ng/pouch) and then increased to approximately 3 ng/pouch within 3 days upon induction of inflammation. Together, these results point at a role for IL-6 in the early stage of inflammation, whereas sIL-6R appears to be important rather at later stages. In non-inflamed air pouches IL-6 as well as sIL-6R were below the detection limit.

3.3.2 Levels of transgenic sgp130Fc in the inflamed air pouch

As mentioned above, for an efficient inhibition of IL-6 transsignaling a molar excess of sgp130 is essential. When using the air pouch model, it is important that sufficient amounts of sgp130Fc protein are present at sites of inflammation. Therefore, levels of sgp130Fc in inflamed air pouches of sgp130Fc transgenic animals were determined by ELISA.

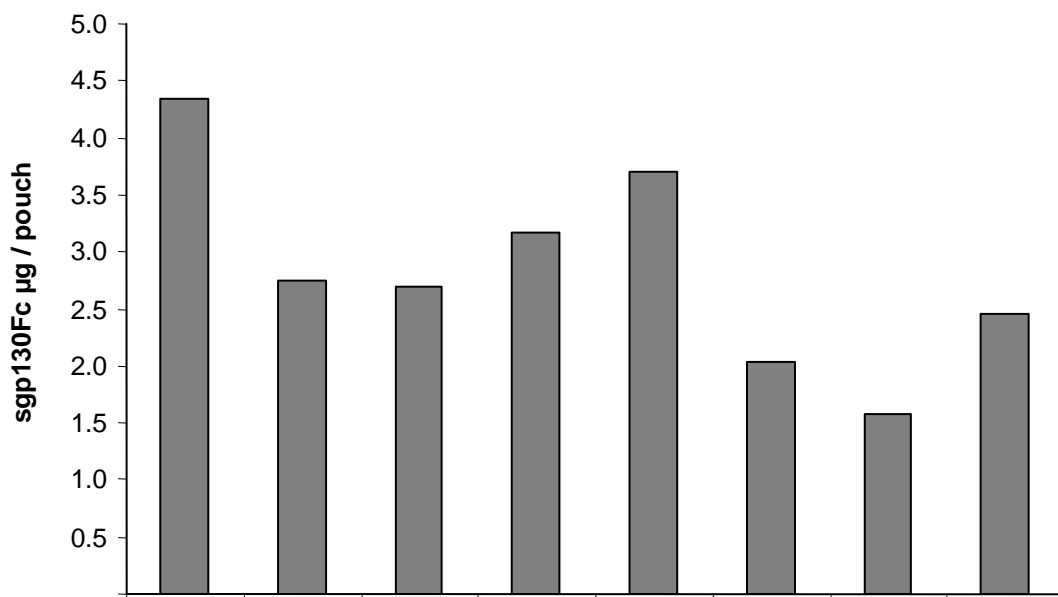


Figure 35: Levels of transgenic sgp130Fc in inflamed air pouches of sgp130Fc transgenic mice. 1% Carrageenan was injected in 6-day-old air pouches and 72 h upon stimulation the levels of sgp130Fc were assessed by ELISA. Each bar represents one sgp130Fc transgenic animal.

The level of endogenous sIL-6R in wildtype animals 72 h upon induction of inflammation was about 3 ng/pouch (Fig. 34B), whereas the concentration of sgp130Fc in the inflamed air

pouch of transgenic animals was 2.8 ± 0.9 $\mu\text{g/pouch}$ (Fig. 35). Therefore, an approximately 250-fold molar excess of sgp130Fc was present in the air pouches. As a 10-fold molar excess of sgp130Fc is sufficient to block IL-6/sIL-6R responses *in vitro* (55), the sgp130Fc levels in inflamed air pouches of sgp130Fc transgenic mice should be high enough to effectively inhibit endogenous IL-6-transsignaling.

3.3.3 Impaired leukocyte recruitment during acute inflammation in sgp130Fc transgenic mice

During acute inflammation, leukocytes (mainly PMNs and monocytes/macrophages, at later stages T and B lymphocytes) migrate from blood vessels into the inflamed tissue (in this case the air pouch). Inflammation was induced by injection of 1% Carrageenan into the air pouches of 8-10 weeks old male sgp130Fc transgenic mice and genetically matched wildtype littermates. Infiltrating cells were counted and then analyzed by Flow Cytometry, using fluorescence-conjugated antibodies specific for neutrophil granulocytes, macrophages, T and B cells.

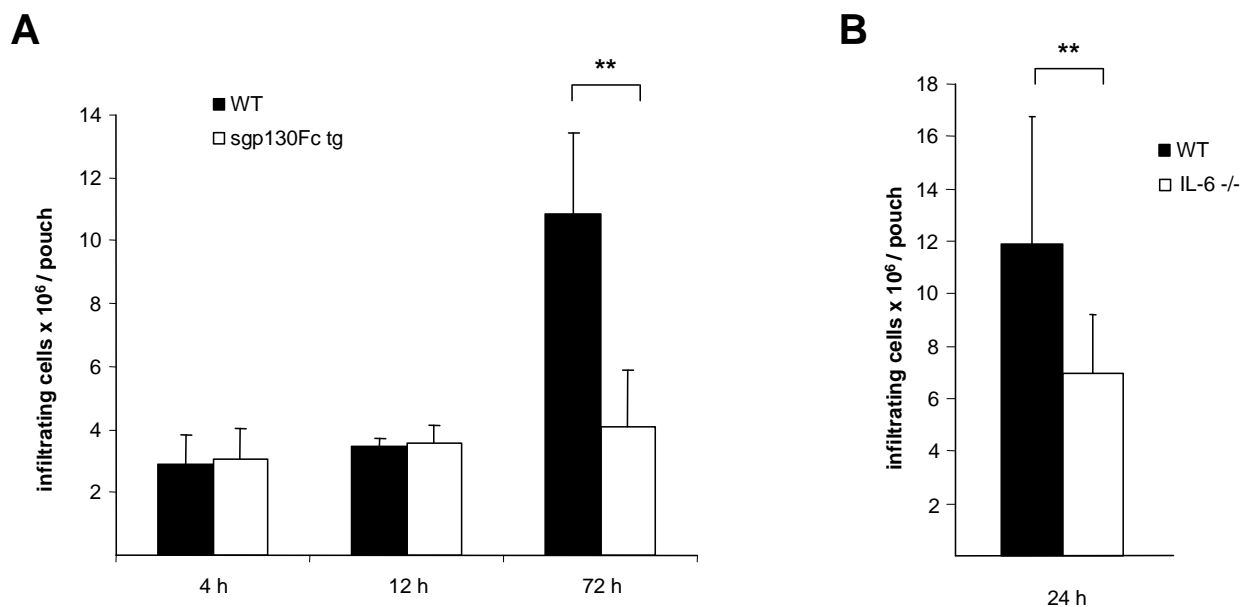
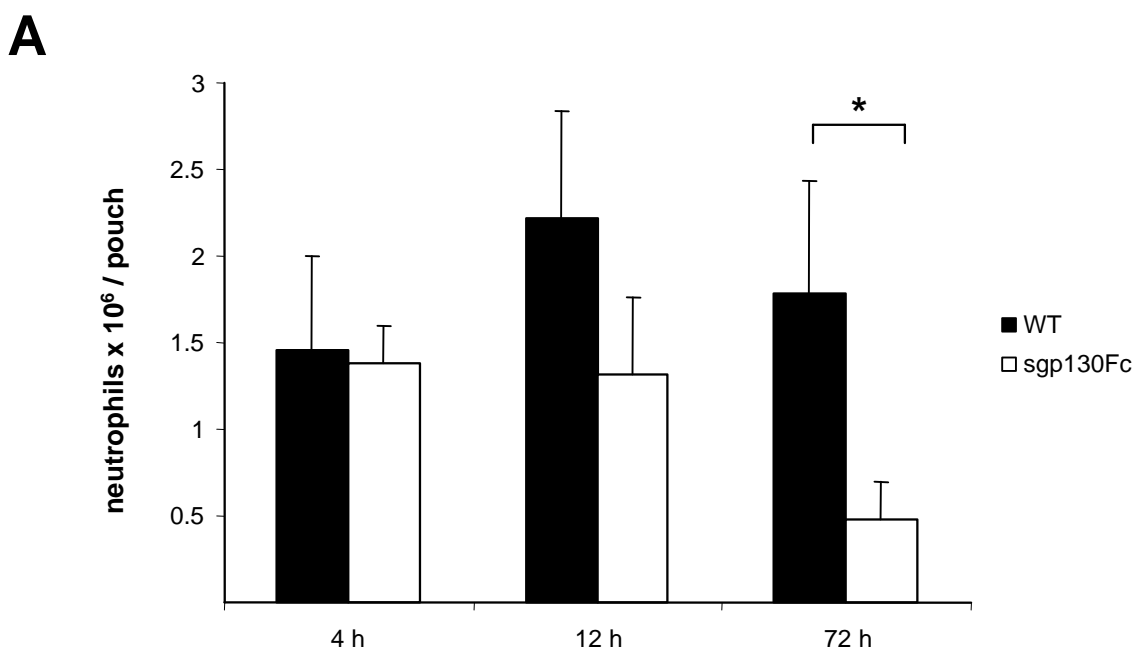


Figure 36: (A) Quantitative Flow Cytometry analysis showing inflammatory cells infiltrating the air pouch 4 h, 12 h and 72 h after injection of 1% Carrageenan. Comparison of homozygous sgp130Fc transgenic and wildtype animals (WT). 4-7 mice per group. Data are represented as mean values \pm SD (**= $p < 0.01$). (B) Total leukocytes infiltrating the air pouch 24 h after 1% Carrageenan challenge. Comparison of IL-6-knock out (IL-6^{-/-}) and wildtype mice. Cells were counted after staining with erythrosine. 5-17 mice per group. Data taken from (96).

Three time points (4 h, 12 h and 72 h) were investigated. The numbers of total cells infiltrating the air pouch were roughly the same 4 h and 12 h after induction of inflammation (about $3 - 3.5 \times 10^6$ infiltrating cells/pouch). Furthermore, no major differences in the number of infiltrating cells between sgp130Fc transgenic and wildtype animals were observed for the two early time points. When analyzing the 72 h time point, it turned out that the number of total infiltrating cells was significantly reduced in sgp130Fc transgenic mice as compared to wildtype mice. Whereas the cell number in wildtype animals increased to 10.9×10^6 /pouch, the inflammatory reaction in the sgp130Fc transgenic mice was markedly suppressed, as no considerable increase in total cell number could be detected (compared to the 4 h and 12 h time points) (Fig. 36A). This is consistent with the work of Romano et al. (96), who used the same air pouch model and showed that in IL-6-deficient mice leukocyte accumulation was significantly impaired (Fig. 36B). In this respect, the results obtained with sgp130Fc transgenic animals revealed that impaired leukocyte accumulation during acute inflammation was only due to IL-6-transsignaling, and not classical IL-6-signaling.

It has been previously shown that the response to Carrageenan is a classical acute inflammation dominated by neutrophil granulocytes (or neutrophils) followed by a mononuclear phase (22). The numbers of both neutrophils and macrophages were assessed by Flow Cytometry at 4 h, 12 h and 72 h after Carrageenan injection in sgp130Fc transgenic and wildtype mice (Fig. 37).



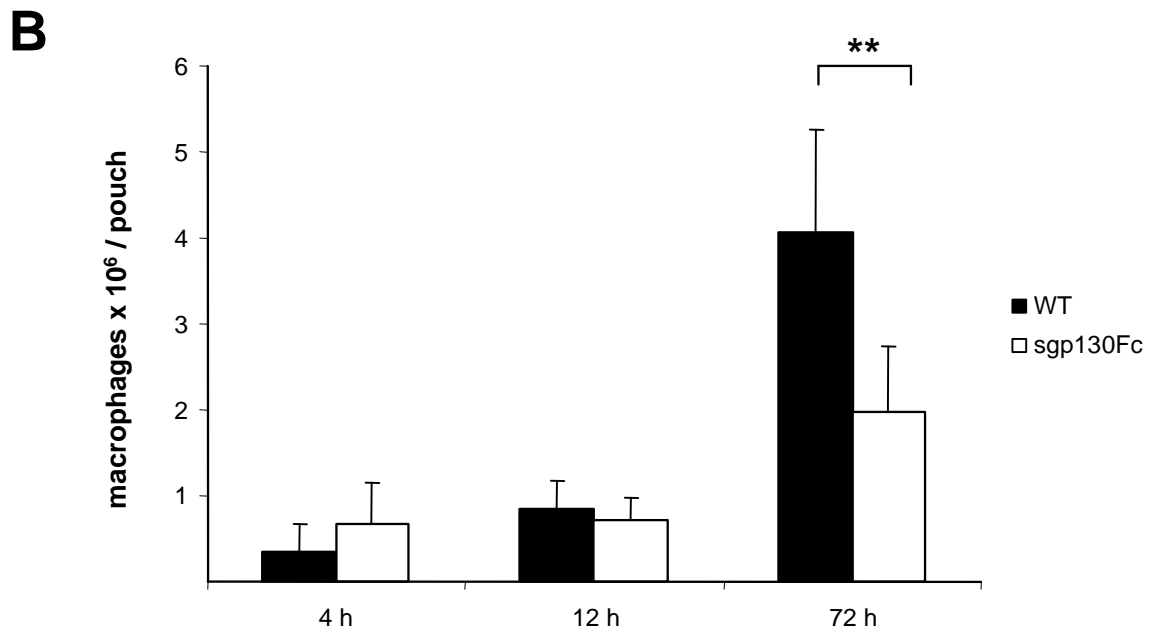


Figure 37: Neutrophils (A) and macrophages (B) infiltrating the air pouch 4 h, 12 h and 72 h after injection of 1% Carrageenan as measured by Flow Cytometry. 4-7 mice per group. Data are represented as mean values \pm SD (**= $p < 0.01$, *= $p < 0.05$).

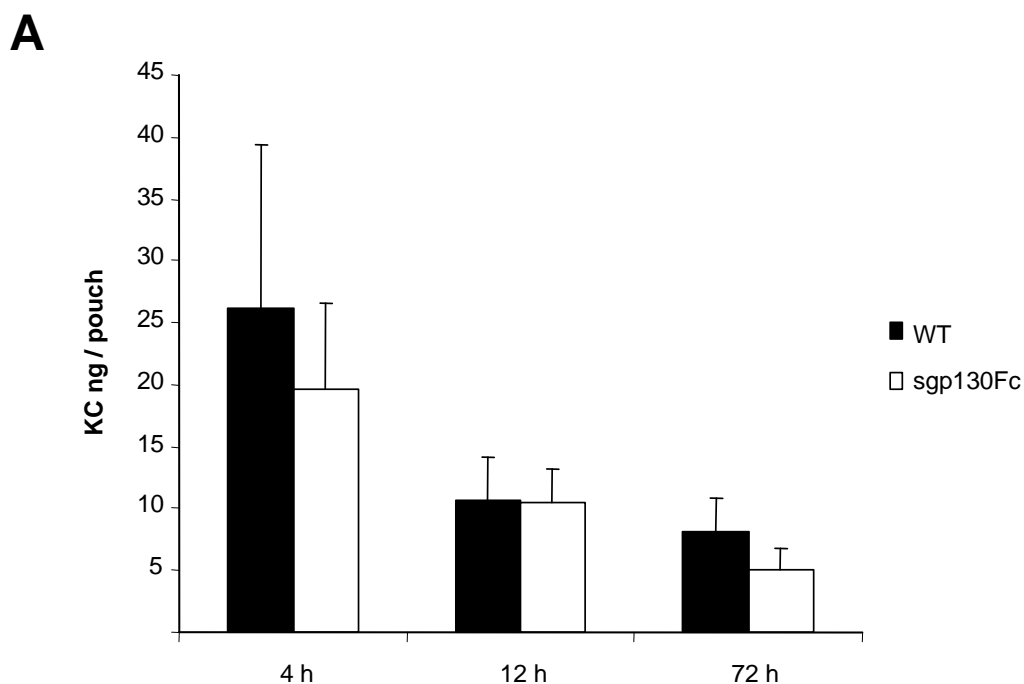
In agreement with previous reports, in wildtype mice neutrophils were the dominating cell type in the inflammatory infiltrates at the early time points (4 h and 12 h). However, during a later stage of inflammation (72 h) macrophages were the predominant leukocyte subpopulation with cell numbers reaching 4×10^6 /pouch ($> 40\%$ of total cells).

In sgp130Fc transgenic mice the proportion of neutrophils in the air pouch was initially comparable with wildtype animals, but then drastically decreased at later time points, whereas the number of neutrophils in wildtype animals peaked at 12 h and slightly declined 72 h after induction of inflammation (Fig. 37A). In terms of infiltrating macrophages no significant differences were observed between sgp130Fc transgenic mice and wildtype animals during the onset of inflammation (4 h and 12 h), with the proportion of macrophages being comparatively low in both groups. In contrast, 72 h upon Carrageenan injection the number of macrophages was strongly reduced in sgp130Fc transgenic mice suggesting that inhibition of IL-6-transsignaling led to impaired macrophage recruitment in these animals (Fig. 37B).

The number of total cells migrated in response to PBS alone was similarly low in sgp130Fc transgenic and wildtype animals at 72 h ($< 0.9 \times 10^6$ /pouch, not presented). No T or B cells were detected in the inflamed air pouch throughout the course of this experiment.

3.3.4 Reduced levels of the CC chemokine MCP-1 during acute inflammation in sgp130Fc transgenic mice

Numerous *in vitro* studies have shown that resident cells (such as fibroblasts, smooth muscle cells and epithelial cells) as well as infiltrating leukocytes are involved in the evolution of the inflammatory response (11). Both cell types may synthesize CXC or CC chemokines after stimulation by proinflammatory cytokines, which in turn recruit granulocytes or monocytes/macrophages to the site of inflammation, respectively. In the murine air pouch model the exudates, which were generated after injection of an inflammatory stimulus, can be easily collected and further analyzed. In this study we focussed on the CXC chemokine KC, which is the murine homologue of human Gro α , and the CC chemokine monocyte chemoattractant protein-1 (MCP-1). KC mediates the recruitment of neutrophils, while MCP-1 directs monocytes/macrophages to the inflammatory site (49). The amounts of both chemokines in the inflamed air pouches of sgp130Fc transgenic and wildtype mice were measured using sandwich ELISA (Fig. 38).



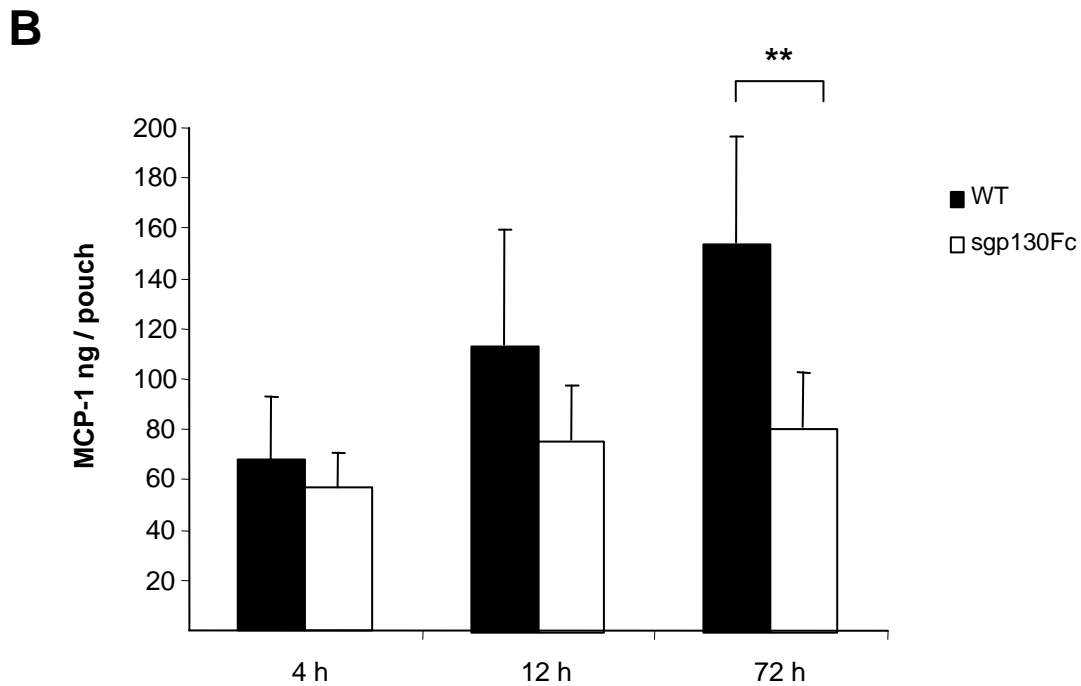


Figure 38: Levels of endogenous KC (A) and MCP-1 (B) in inflamed air pouches of sgp130Fc transgenic and wildtype mice 4 h, 12 h and 72 h following Carrageenan challenge. KC and MCP-1 concentrations were determined by ELISA. 4-7 mice per group. Data are represented as mean values \pm SD (**= $p < 0.01$).

In wildtype animals KC levels peaked at 4 h after induction of inflammation and rapidly decreased during the later stages of inflammation. Conversely, the amounts of MCP-1 were moderately increasing during the onset of inflammation, whereas the highest levels were measured 72 h after induction of inflammation. These results reflect the fact that neutrophils were the first cells to enter the site of an inflammation, which were later to be replaced by monocytes/macrophages.

With respect to the KC levels sgp130Fc transgenic mice showed no significant difference compared to wildtype animals, although the amounts of KC in the transgenic animals were slightly reduced 4 h and 72 h after induction of inflammation. By contrast, a clear difference could be observed regarding the levels of MCP-1 in the inflamed air pouch. Whereas the amounts of MCP-1 in wildtype animals steadily increased and peaked at 72 h upon injection of Carrageenan, the MCP-1 levels in sgp130Fc transgenic animals were slightly lower at 4 h and then plateaued during the course of inflammation. These results clearly show that the observed deficit in macrophage recruitment is due to reduced MCP-1 levels, since MCP-1 is the most important chemokine involved in the attraction of macrophages. Additionally, this is

consistent with the work of Romano et al. (96), who showed that in IL-6-deficient mice both leukocyte accumulation and MCP-1 production were impaired.

3.3.5 Soluble IL-6R is released from infiltrating neutrophils (neutrophil depletion)

The cellular source of sIL-6R in the air pouch model as well as in any other inflammation model is not known. Since the lining cells of the air pouch are very unlikely to express IL-6R (96) and a positive correlation between infiltrating leukocytes and the amount of sIL-6R was found in a murine peritonitis model (49), it is therefore tempting to speculate that either infiltrating neutrophils or macrophages release their membrane-bound IL-6R upon migration into the inflamed tissue.

Mice injected i.p. with purified Ly6GC mAB, which recognize a surface marker on mature murine granulocytes (Gr-1), experienced a profound depletion of neutrophils in the bloodstream and the air pouch (20, 36). To generate neutropenic animals, 8-10 weeks old male C57BL/6 wildtype mice were challenged i.p. with 100 µg of Ly6GC mAB 18 h before injection of 1% Carrageenan. Cell numbers of neutrophils and macrophages were determined by Flow Cytometry, while levels of sIL-6R were assessed by ELISA in the inflamed air pouches 24 h and 72 h after induction of inflammation.

As already seen in the FSC/SSC plot (Fig. 39A), a distinct population of neutrophils emerged 24 h after Carrageenan injection, which disappeared when the animals were treated with the depleting antibody before Carrageenan challenge. The quantitative Flow Cytometry analysis, shown in Fig. 39B, revealed that 24 h after induction of inflammation no neutrophils were present in the inflamed air pouch of neutropenic animals. 72 h after Carrageenan injection neutrophils were hardly detectable. These results indicate that in inflamed wildtype mice neutrophil depletion was successful.

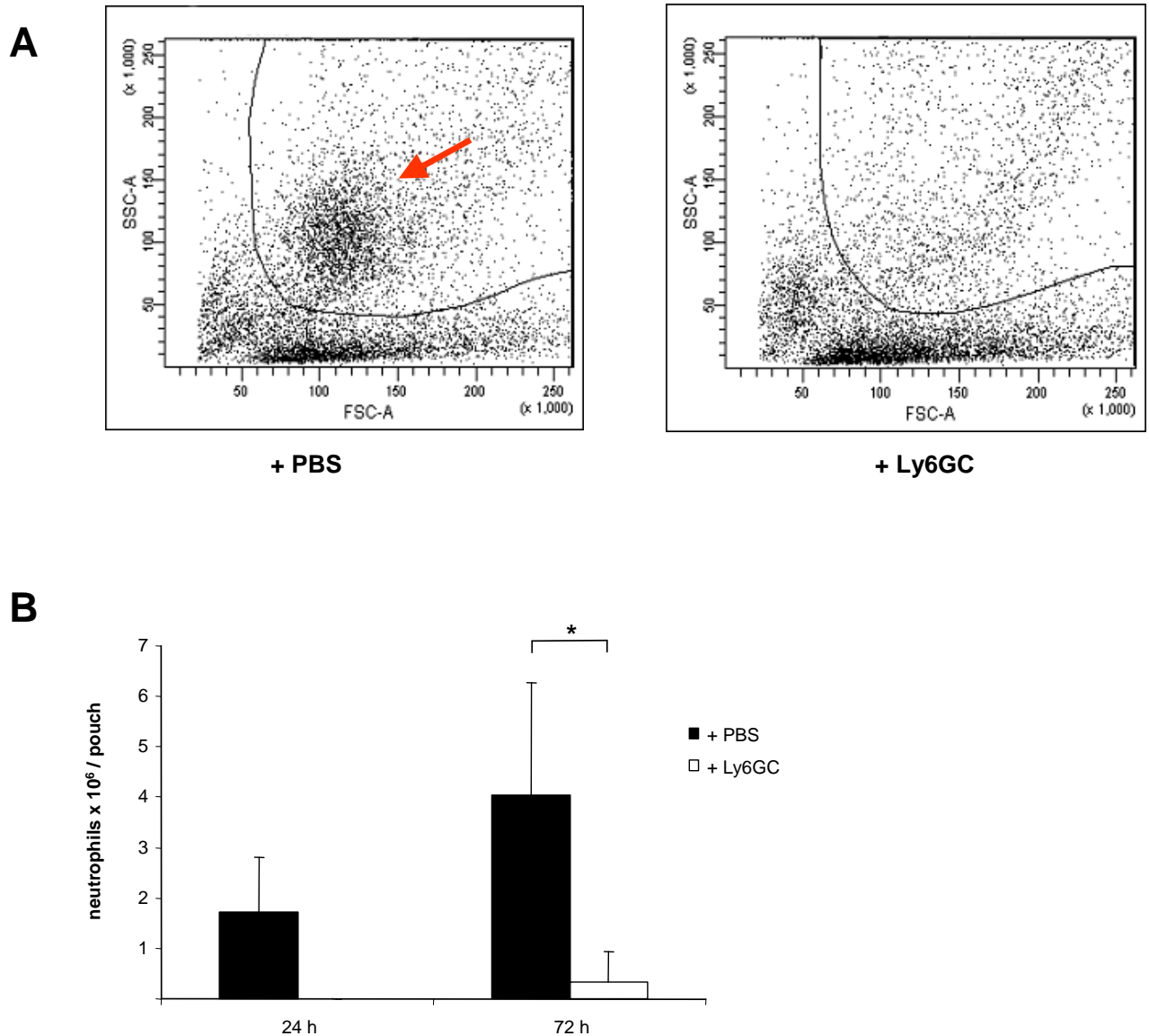


Figure 39: Flow Cytometry analysis showing that in inflamed air pouches of C57BL/6 wildtype mice neutrophil depletion was successful. 18 h before injection of Carrageenan animals were administered i.p. with 100 μ g of a neutrophil depleting mAB (Ly6GC) or PBS. (A) FSC/SSC plot. 24 h after induction of inflammation a distinct neutrophil population was visible in PBS treated mice (left), which disappeared in neutropenic animals (right). (B) Quantitative Flow Cytometry analysis revealed that neutrophils were hardly detectable in inflamed air pouches 24 h and 72 h upon induction of inflammation. 4 mice per group. Data are represented as mean values \pm SD (*= $p < 0.05$).

With respect to the levels of sIL-6R no difference was observed in the inflamed air pouches of PBS treated and neutropenic animals 24 h after Carrageenan injection. In contrast, 72 h after

induction of inflammation a strong increase in the amount of sIL-6R was noticed in PBS treated mice, whereas in neutropenic mice no appreciable increase was found (Fig. 40). These results clearly show, that sIL-6R was released from neutrophils, that migrated into the inflamed air pouch.

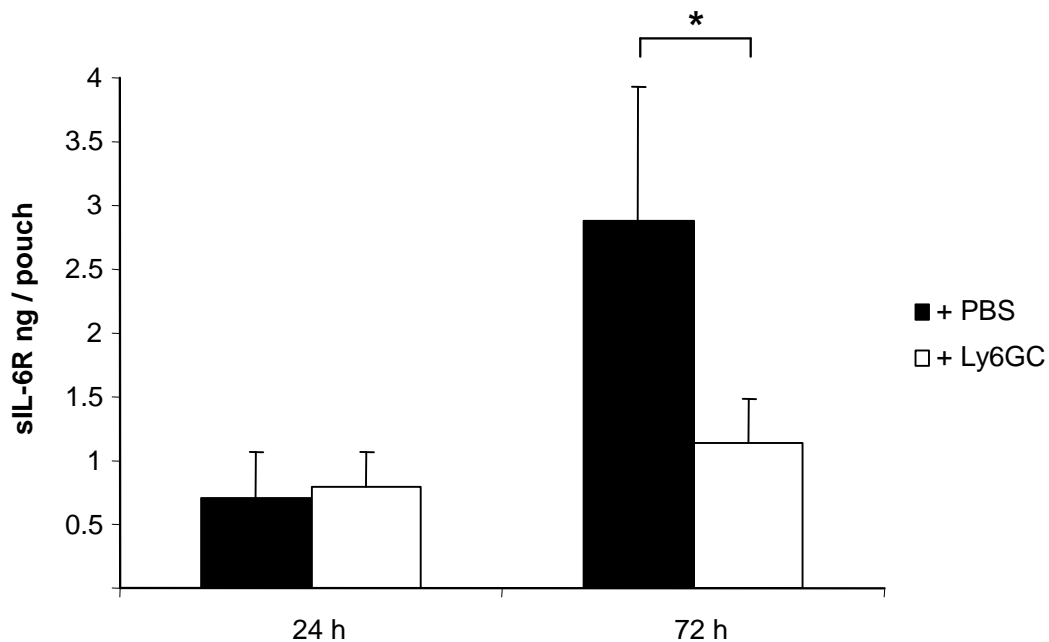


Figure 40: sIL-6R was released from infiltrating neutrophils. Levels of sIL-6R were determined by ELISA 24 h and 72 h after injection of Carrageenan in PBS and Ly6GC mAB treated C57BL/6 wildtype mice. 4 mice per group. Data are represented as mean values \pm SD (*= $p < 0.05$).

The proportion of macrophages in the inflamed air pouches of control and neutropenic animals 24 h after injection of Carrageenan was not modified (Fig. 41). However, a drastic decrease in macrophage numbers was observed in neutropenic animals compared to control mice after 72 h of treatment, suggesting a neutrophil derived factor being implicated in macrophage recruitment during the later stages of inflammation.

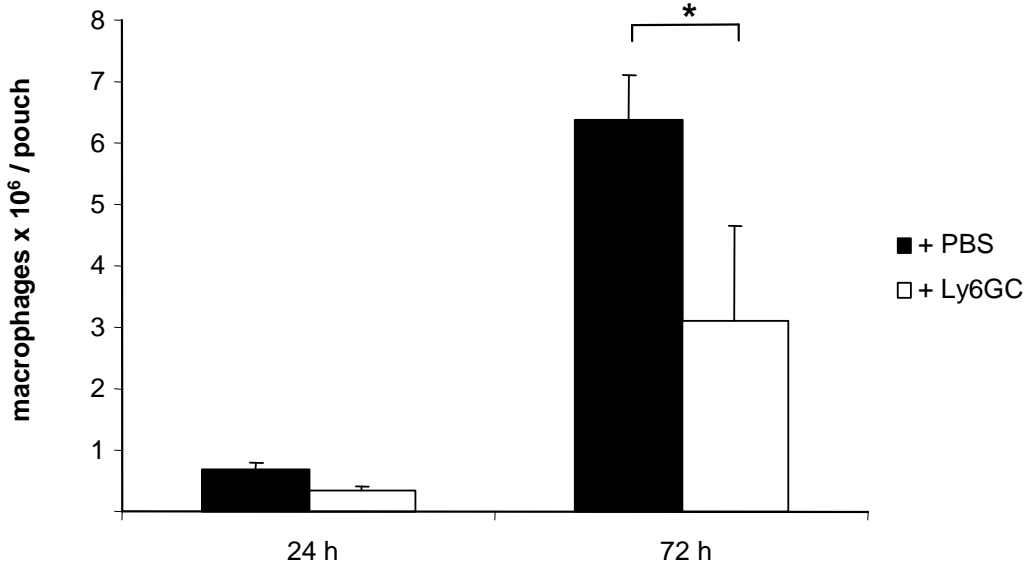


Figure 41: Macrophage recruitment was impaired in neutropenic mice. Quantitative Flow Cytometry analysis showing the number of macrophages 24 h and 72 h after injection of Carrageenan in PBS and Ly6GC mAB treated C57BL/6 wildtype mice. 4 mice per group. Data are represented as mean values \pm SD (*= p < 0.05).

4 Discussion

4.1 Generation of sgp130Fc transgenic mice

Sgp130 is the natural inhibitor of IL-6-transsignaling and selectively blocks IL-6 responses mediated by the sIL-6R, whereas IL-6 responses via the membrane-bound IL-6R remain unaffected. Soluble IL-6R mediated signaling has been implicated in many pathophysiological conditions including rheumatoid arthritis (85), peritonitis (49), colon cancer (7) and inflammatory bowel disease (4). Administration of recombinant sgp130Fc in animal models of the latter diseases has proven effective to ameliorate the clinical score in the respective murine disease models.

In order to examine the pathophysiological role of sIL-6R mediated responses *in vivo*, transgenic mice were generated in this project, which overexpress human sgp130Fc from a liver-specific promoter. In this respect it is important to consider the species specificity of sIL-6R and the other components of the IL-6 receptor system (Fig. 42). It is long known that there is a strict species specificity for the interaction between IL-6 and the IL-6R. Human IL-6 binds to both, human and mouse IL-6R, whereas murine IL-6 only binds to the murine receptor (118). In contrast, murine sIL-6R is able to associate with murine and human IL-6 as well as murine and human sgp130 (55). Endogenous IL-6-transsignaling responses in mice, which involve both murine IL-6 and murine sIL-6R, can therefore be inhibited by human sgp130.

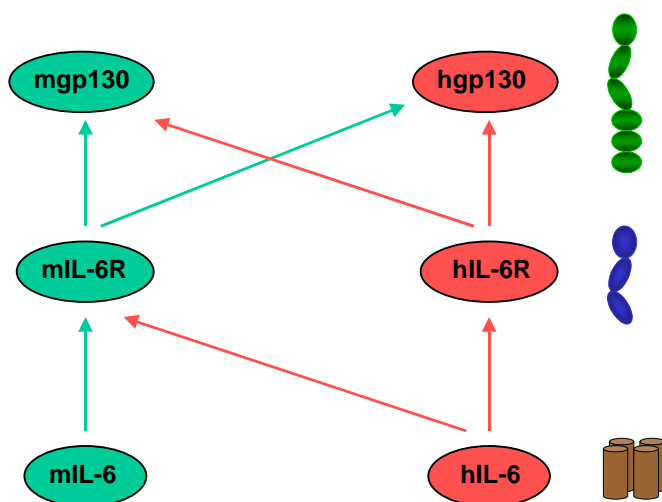


Figure 42: Species specificity within the IL-6-receptor-system. Human IL-6 (hIL-6) can bind both human (hIL-6R) and murine IL-6R (mIL-6R), whereas murine IL-6 (mIL-6) only binds murine IL-6R. The murine IL-6R, like human IL-6R, can associate with both human (hgp130) and murine gp130 (m gp130).

Sgp130Fc is a fusion protein consisting of the extracellular portion of gp130 connected to the constant region of a human IgG1 heavy chain (Fc part). When considering its inhibitory capacity the dimerized sgp130Fc turned out to be about 10-fold more active than monomeric sgp130, which is most likely due to the chimeric character of sgp130Fc. By employing co-precipitation experiments and fluorescence resonance energy transfer (FRET) it was recently shown that on the living cell gp130 exists as a preformed dimer in the plasma membrane. Addition of ligand further enhances the dimer formation presumably through changes in the spatial orientation of the receptor chains, which in turn leads to receptor activation and triggering of intracellular signaling cascades (40, 115). The availability of a preformed gp130 dimer accelerates the receptor complex assembly, since two gp130 monomers do not need to find each other on the plasma membrane to form a signaling competent receptor complex. Therefore, sgp130Fc reflects the situation on the living cell, which is most likely the reason for the higher activity of sgp130Fc compared to the monomeric protein.

Taking into account both the dimeric character of sgp130Fc and the binding promiscuity of murine sIL-6R towards IL-6 and sgp130, transgenic mice expressing human sgp130Fc are an ideal model system to analyze the consequences of murine IL-6-transsignaling and represent an “IL-6-transsignaling knockout” in mice.

In general, blocking of sIL-6R mediated responses requires a molar excess of sgp130, since sgp130 has to compete with the surface expressed counterpart for the IL-6/sIL-6R complex (55). Hence, when considering the inhibition of IL-6-transsignaling in mice, it is mandatory that transgenic sgp130 is present in sufficient amounts to effectively block the complex of murine IL-6 and sIL-6R.

In this project, transgenic mice were generated, which express human sgp130Fc under the transcriptional control of the PEPCK promoter. This promoter is mainly active in liver and kidney (5, 70). Since the liver exhibits a high synthetic capacity and the PEPCK promoter is considered to be comparatively strong, transgenic proteins expressed under the PEPCK promoter have been shown to accumulate in high amounts in the circulation with serum levels of the transgene being in the low microgram range (70, 122). For instance, sIL-6R transgenic and IL-6/sIL-6R double transgenic mice show serum concentrations of 4-8 μg sIL-6R/ml serum (89, 106). In contrast, sgp130Fc transgenic mice of the “first generation” showed transgene serum levels of ≤ 0.55 $\mu\text{g}/\text{ml}$ (see Fig. 18), which is far below the transgene serum concentration observed for sIL-6R transgenic mice. There are several reasons, which could contribute to this comparably low expression of sgp130Fc. For example, gene silencing effects have been observed when the transgene integrated into heterochromatic regions of the

mouse genome such as the centromere or telomere or their borders (27, 30, 71). But since all four independent transgenic lines (L1, L3, L4 and L6) showed similar weak transgene expression, this explanation is rather unlikely, even though the exact transgene integration sites have not been determined. It has also been reported that the presence of multiple copies of a transgene within a concatameric array can have a repressive effect upon gene expression in mammalian systems (repeat-induced gene silencing), with a reduction in copy number resulting in a marked increase in expression of the transgene (37, 69). Furthermore, DNA methylation of CpG-rich sequences may lead to reduced transgene expression (15).

Phosphoenolpyruvate-carboxykinase (PEPCK) is the key regulatory enzyme of hepatic gluconeogenesis and feeding the animals a carbohydrate low diet resulted in stimulation of the PEPCK promoter, which controlled sgp130Fc expression at the transcriptional level (70). Nevertheless, serum concentrations of sgp130Fc could not substantially be elevated (sgp130Fc levels were estimated to be in the range of 1-2 $\mu\text{g/ml}$ serum after the carbohydrate low diet) and the relatively low sgp130Fc serum levels in the first transgenic mice failed to inhibit the Hyper-IL-6-induced acute phase response. Therefore, a new generation of transgenic animals was generated, which express sgp130Fc from a codon-optimized cDNA. Codon-optimization of a defined cDNA leads to increased mRNA stability and enhanced translational efficiency. In addition, the optimized sgp130Fc cDNA was inserted behind the 2nd β -globin intron into the expression vector, whereas the protein coding region in the original sgp130Fc transgenic mice was located in front of the intron. It has been previously reported that the position of the heterologous intron within the expression vector can be crucial for effective transgene expression (86). Indeed, a more than 50-fold increase in the sgp130Fc serum concentration was observed in homozygous animals of the “second generation” when compared to homozygous animals of the “first generation” of sgp130Fc transgenic mice (0.55 \rightarrow 34.3 $\mu\text{g/ml}$ serum). Transfection experiments in HepG2 cells revealed that both cDNA optimization and the effect of the intron position contributed to the enhanced transgene expression. Interestingly, neither cDNA optimization nor the change of the intron position alone resulted in an appreciable increase in transgene expression with sgp130Fc being hardly detectable in the corresponding HepG2 cell supernatants (Fig. 25).

To prove that the sgp130Fc protein produced from transgenic mice is active, two different approaches were taken. In the first experiment serum from transgenic mice was mixed with Hyper-IL-6, a fusion protein of IL-6 and the ligand binding domain of IL-6R (31). It has been previously shown that recombinant sgp130Fc is able to bind Hyper-IL-6 and to inhibit Hyper-IL-6 mediated proliferation of gp130 expressing hematopoietic progenitor cells (55). It clearly

turned out that sgp130Fc, derived from the serum of transgenic mice, can precipitate recombinant Hyper-IL-6 indicating that the transgenic protein is active.

The acute phase response, which is elicited a short time after injuries or the onset of infections, is characterized by the coordinated synthesis of so-called acute phase proteins (APPs) by hepatocytes in the liver (101). Serum amyloid A (SAA) is one of the major APPs in mice and it is upregulated up to 1,000-fold in response to various stimuli, among them bacterial lipopolysaccharides and cytokines such as IL-1, IL-6 and TNF α (1).

Whereas gp130 tissue expression is ubiquitous, the IL-6R is expressed only on a few cell types, including hepatocytes and some leukocyte subpopulations (T and B lymphocytes, neutrophils, monocytes/macrophages). The number of gp130 molecules on the cell surface is believed to be relatively constant, while the amount of IL-6R differs from one cell type to another. Hepatocytes express fewer IL-6R molecules on their surface than gp130 and can respond to IL-6 alone, but this response can be enhanced by the addition of the sIL-6R (90). Injection of Hyper-IL-6 into the peritoneal cavity of wildtype mice therefore led to a prolonged acute phase response. In addition, the acute phase reaction turned out to be more pronounced as compared to IL-6 (88), which is due to the fact that 10 times more gp130 than IL-6R molecules are located on the hepatocyte surface (90). As assessed by Northern blotting of SAA mRNA, in sgp130Fc transgenic mice of the “second generation” the acute phase response was significantly reduced upon intraperitoneal injection of Hyper-IL-6 when compared to wildtype animals (Fig. 32). This result first demonstrates, together with the Hyper-IL-6 immunoprecipitation experiment, that transgenic sgp130Fc is functionally active. In addition, the experiment shows that the amounts of sgp130Fc in the “second generation” of transgenic mice were sufficient to suppress the acute phase response induced by Hyper-IL-6. This is in contrast to the first sgp130Fc mice, which did not produce enough sgp130Fc to inhibit the Hyper-IL-6-induced acute phase.

As mentioned above, the PEPCCK promoter, which drives the expression of sgp130Fc in the transgenic animals, has been shown to be mainly active in liver and kidney (63, 70). Consistently, in sgp130Fc transgenic animals a strong expression of sgp130Fc was observed in liver and kidney. However, appreciable amounts of sgp130Fc mRNA were also found in lung and muscle, which is in contrast to some previous reports (43, 70).

4.2 Role of sIL-6R during acute inflammation (air pouch model)

Inflammation is the response of living tissue to damage, microbial infections or other immunologic stimulation. The local inflammatory reaction is characterized by an increased blood supply to the tissue "in danger" and enhanced vascular permeability with white blood cells migrating out of the capillaries into the inflamed tissue. The increased capillary permeability is thereby provoked by retraction of the endothelial cells. In the earliest stages of inflammation neutrophils are particularly prevalent, at later stages monocytes, macrophages and lymphocytes (specific subsets of T-cells and B-cells) appear at the site of injury/infection. Neutrophils and macrophages mount a rapid, non-specific phagocytic response by taking up bacteria and cellular debris, whereas lymphocytes are associated with antigen-specific and more tightly regulated immune responses. The highly efficient process of cellular influx to inflammatory sites is mediated by cytokines, chemokines, products of the plasma enzyme systems (complement, the coagulation clotting, kinin and fibrinolytic pathways), lipid mediators (prostaglandins and leukotrienes) released from different cells, and by vasoactive substances secreted from mast cells, basophils and platelets. When leukocytes have arrived at an infected area, they release mediators which control the later accumulation and activation of other cells (50). The major proinflammatory cytokines, which are responsible for early responses, are IL-1, TNF α and IL-6. Most of them are released from tissue-resident macrophages and activate endothelial cells to upregulate the receptors VCAM-1, ICAM-1, E-selectin, and L-selectin for various immune cells and to produce an array of chemokines that chemoattract inflammatory cells (3, 46). Cleavage and activation of IL-1 β by so-called inflammatory caspases (i.e. caspase-1 and caspase-5) proceeds in a large multiprotein complex that has been termed inflammasome (92).

There are several lines of evidence that sIL-6R signaling is involved in the control of leukocyte recruitment during inflammation (73, 96). In particular, it has been proposed that the transition from the initial neutrophilic stage to the more sustained influx of mononuclear leukocytes strongly relies on the presence of IL-6 and the soluble IL-6R (Fig. 43). Thereby soluble IL-6R is most likely released from infiltrating neutrophils, since sIL-6R concentrations in early inflammatory exudates strongly correlate with leukocyte numbers. Combined IL-6 and sIL-6R then triggers endothelial/mesothelial cells to synthesize the chemokines MCP-1 and MCP-3, which attract monocytes and macrophages, whereas secretion of IL-8 and GRO α is concurrently suppressed. The latter chemokines mediate the

recruitment of neutrophils to the site of inflammation. Mesothelial as well as endothelial cells do not express the cognate IL-6R, which infers that these cells are unresponsive to IL-6 itself and therefore necessitate additional sIL-6R for signal transduction (49, 96).

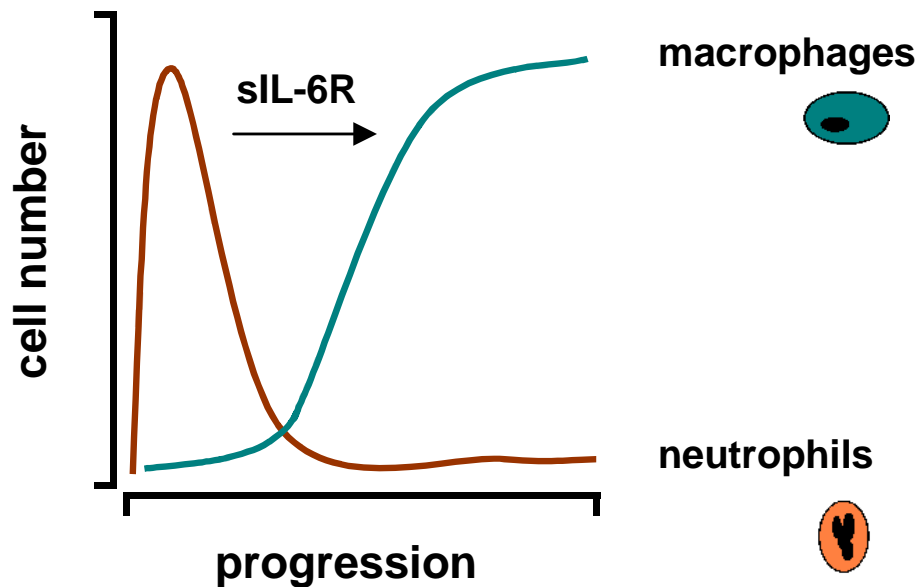


Figure 43: IL-6-transsignaling differentially regulates the switch between the initial neutrophilic stage of inflammation and the later, more sustained influx of mononuclear cells.

In previous animal studies dealing with the impact of IL-6-transsignaling on inflammation either IL-6 knock-out mice were employed, in which sIL-6R mediated signaling was restored by administration of recombinant Hyper-IL-6 (alternatively IL-6 in combination with sIL-6R), or wildtype mice were challenged with doses of recombinant sgp130Fc to selectively block sIL-6R responses. Since the sgp130Fc transgenic mice, which were generated in this work, permanently produce high levels of endogenous sgp130Fc, these animals are an elegant tool to effectively inhibit IL-6-transsignaling *in vivo*. Especially, sgp130Fc transgenic mice represent the ideal model system to investigate the influence of sIL-6R on the development of chronic inflammatory diseases, such as rheumatoid arthritis or inflammation-induced colon cancer, which would require the presence of sgp130 over long periods.

In this work the murine air pouch model was used to investigate the role of sIL-6R signaling in the evolution of an acute inflammatory response. Injection of Carrageenan into dorsal air pouches of mice resulted in a local inflammatory reaction, which has been monitored over a

time period of 3 days. In the initial phase of inflammation (4 h and 12 h after induction) high levels of IL-6 were measured in the pouch exudate, which declined thereafter, whereas considerable amounts of sIL-6R were found only at later time points (48 h and 72 h, Fig. 34). This leads to the suggestion that IL-6 plays an important role in the early onset of inflammation, while sIL-6R mediated signaling is involved rather at a later stage of an inflammatory insult.

The air pouch is a highly vascularized compartment and contains copious vessels with features of high endothelial venules (21, 96). Endothelial cells play an important, active role in inflammatory processes and secrete high amounts of IL-6 in response to proinflammatory signals, including microbial products, IL-1 and TNF α (51, 93, 108). As noted previously, endothelial cells lack membrane-bound IL-6R and therefore do not respond to IL-6 alone. Since endothelial cells express gp130, the presence of sIL-6R would render them responsive to IL-6. Given that a molar excess of sgp130 is needed to successfully disturb sIL-6R activities, it is of importance that sufficient amounts of sgp130Fc are available in the air pouch. A molar excess of approximately 250-fold could be detected in the pouch fluid when comparing transgenic sgp130Fc and endogenous sIL-6R levels (Fig. 35). It has been shown *in vitro* that a 10-fold excess of sgp130Fc is enough to block sIL-6R mediated cell proliferation. Therefore the sgp130Fc/sIL-6R ratio found in inflamed air pouches of sgp130Fc transgenic mice was sufficient to inhibit murine IL-6-transsignaling.

During the onset of inflammation, the Carrageenan induced response was dominated by neutrophils, which infiltrated the air pouch as early as 4 h after injection, followed by a delayed mononuclear phase (Fig. 37). When considering the number of total cells infiltrating the air pouch, it turned out that the overall inflammatory reaction was strongly reduced in sgp130Fc transgenic mice, since cell numbers did not considerably increase during development of inflammation as compared to wildtype animals (Fig. 36). Neutrophil numbers in sgp130Fc transgenic animals were similar to wildtype mice 4 h after induction of inflammation, but strongly decreased during later stages of inflammation, whereas neutrophils in wildtype animals only moderately declined. Macrophage numbers were roughly the same in sgp130Fc transgenic and wildtype mice 4 h and 12 h after stimulation. In wildtype animals a substantial rise of mononuclear cells was observed 72 h after induction, while in the presence of transgenic sgp130Fc macrophage numbers remained constant during the course of inflammation (Fig. 37). At the chemokine level the reduced accumulation of monocytes/macrophages in sgp130Fc transgenic mice was paralleled by a diminished secretion of MCP-1 into the pouch (Fig. 38). This is in agreement with the work of Romano et

al. (96), who also employed the murine air pouch model of acute inflammation showing that in IL-6 knockout mice both leukocyte recruitment and MCP-1 secretion was significantly reduced. It has also been shown that at the cellular level the stimulation of mesothelial and endothelial cells with the IL-6/sIL-6R complex resulted in a strong upregulation of MCP-1. Furthermore, i.p. administration of Hyper-IL-6 in a murine peritonitis model led to an enhanced MCP-1 response (49, 56, 96). Although neutrophil numbers in sgp130Fc transgenic mice were strongly reduced during later stages of inflammation, the KC levels measured in the inflamed air pouches of the transgenic animals did not significantly differ from those found in wildtype mice, suggesting that in this disease model probably other CXC chemokines, such as MIP-2 (the murine homologue of IL-8), were involved in neutrophil recruitment (Fig. 38). Taken together, these results indicate an important role for the IL-6/sIL-6R complex in mediating the switch from the initial neutrophilic to the later mononuclear phase of inflammation, since in sgp130Fc transgenic mice both the accumulation of macrophages and neutrophils was impaired only at later stages in the development of inflammation (which was additionally confirmed by the reduced MCP-1 levels). In contrast, the initial influx of neutrophils, which is commonly regarded as the first step of an inflammatory response, was not affected by sgp130Fc.

The cellular source of sIL-6R in acute as well as chronic inflammatory conditions is not known yet. Endothelial cells, which are probably the most important cells in the evolution of inflammation as they secrete the chemokines that in turn attract the respective inflammatory cells, do not express cognate IL-6R. However, there is strong evidence that infiltrating leukocytes, such as neutrophils and macrophages, substantially contribute to the levels of sIL-6R found in inflammatory exudates. For instance, in rheumatoid arthritis patients a strong correlation exists between the degree of leukocyte infiltration into an arthritic joint and elevated synovial sIL-6R concentrations (23). A similar correlation is observed in early inflammatory exudates of a murine peritonitis model, where increased leukocyte numbers have been associated with elevated sIL-6R levels. Since infiltrating cells at such early stages of inflammation mainly consist of neutrophils, it has been concluded that neutrophils are most likely the main source of released sIL-6R (49). However, direct evidence is still missing that the sIL-6R is liberated from infiltrating neutrophils. In the present work neutropenic mice were generated by i.p. injection of a neutrophil depleting antibody with no neutrophils being detectable in the inflamed air pouch 24 h as well as 72 h following Carrageenan injection. While sIL-6R levels in untreated animals significantly increased 72 h after stimulation, no rise of sIL-6R was observed in neutropenic mice clearly indicating that during acute inflammation

sIL-6R was released from migrating neutrophils (Fig. 40). In addition, macrophage recruitment was impaired in neutropenic animals to a similar extent as seen in *sgp130Fc* transgenic mice suggesting that sIL-6R is the critical neutrophil-associated factor implicated in macrophage accumulation during later stages of inflammation (Fig. 41).

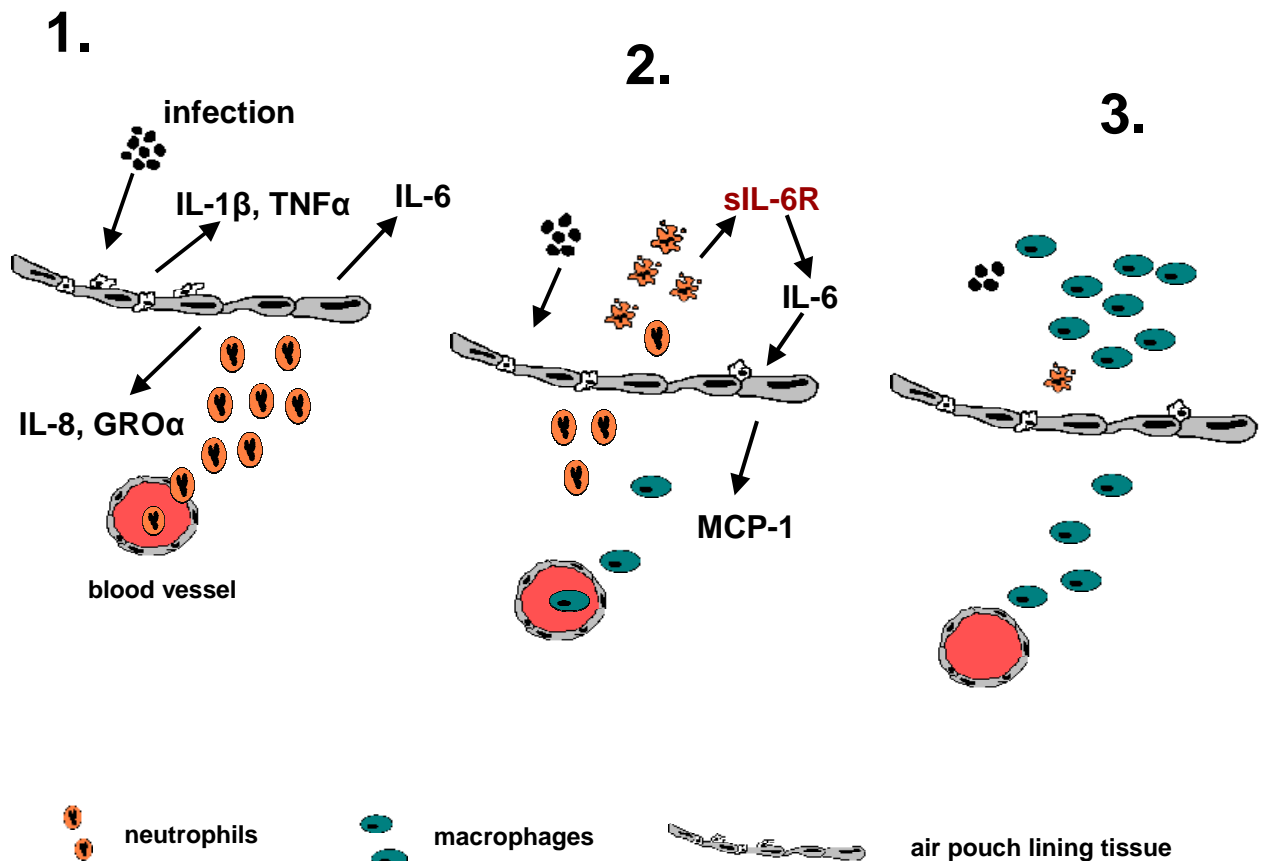


Figure 44: Infection/inflammation stimulates tissue-resident macrophages to secrete the proinflammatory cytokines $\text{TNF}\alpha$ and $\text{IL-1}\beta$, which in turn induce CXC chemokine and IL-6 secretion from endothelial cells (1). Endothelial cells do not express membrane-bound IL-6R and are therefore not responsive towards IL-6 itself. Infiltrating neutrophils shed their IL-6R upon apoptosis and the IL-6/sIL-6R complex stimulates endothelial cells to produce the CC chemokine MCP-1 (2), which subsequently attracts macrophages to the site of inflammation. The replacement of neutrophils by mononuclear cells (3) is an important intermediate step in the resolution of inflammation, which is controlled by the presence of sIL-6R.

This is consistent with previous studies showing that sIL-6R is shed from neutrophils following stimulation with f-Met-Leu-Phe, C-reactive protein and IL-8 as well as $\text{GRO}\alpha$ (49,

53, 73). Very recent data obtained in our laboratory reveal that sIL-6R is shed from apoptotic neutrophils by the metalloprotease ADAM17 (13).

Based on the results presented in this work (utilizing sgp130Fc transgenic and neutropenic mice in an air pouch model of acute inflammation) combined with the data observed with apoptotic neutrophils we propose the following model regarding the role of sIL-6R during inflammation (Fig. 44): The first step in an inflammatory reaction is the release of the pro-inflammatory cytokines IL-1 β , TNF α and IL-6. Endothelial cells do not express IL-6R and are therefore not responsive to IL-6 but are responsive to IL-1 β and TNF α . Activation of endothelial cells leads to the secretion of CXC chemokines and subsequently to the attraction of neutrophils. Apoptotic neutrophils then shed their membrane-expressed IL-6R and the complex of IL-6 and sIL-6R stimulates endothelial cells to produce CC chemokines (i.e. MCP-1) leading to a substitution of neutrophils by monocytes/macrophages. Thus, the transition from the initial neutrophilic to the more sustained mononuclear phase of inflammation is controlled by the presence of sIL-6R which is shed from apoptotic neutrophils (Fig. 43, 44).

4.3 Outlook

In this work transgenic mice were generated, which accumulated sgp130Fc in high amounts in the circulation. The murine air pouch model of acute inflammation was used to initially characterize these animals and it could be demonstrated that sgp130Fc transgenic mice showed a reduced inflammatory reaction in response to Carrageenan challenge.

Sgp130Fc transgenic mice represent an “IL-6-transsignaling knock out” model. Although sgp130Fc transgenic mice showed high serum levels of sgp130Fc, the animals exhibited no obvious phenotypic alterations. It is therefore tempting to speculate that IL-6-transsignaling plays no role in daily life. In contrast, it has been shown that IL-6-transsignaling is involved in the pathogenesis as well as the maintenance of several chronic inflammatory diseases, including inflammatory bowel disease (4), ileitis (72), rheumatoid arthritis (23, 85) and even inflammation-induced colon cancer (7).

It would be therefore of special interest to investigate the impact of permanently high sgp130Fc serum levels on the onset and perpetuation of these inflammatory disorders, since in mice these diseases usually develop over a longer time period and would require the long-term administration of exogenous sgp130Fc. Therefore, continuous inhibition of IL-6-

transsignaling in sgp130Fc transgenic animals will help to clarify the role of sIL-6R mediated signaling in the development of chronic diseases, infections and cancer.

5 Summary

Interleukin-6 (IL-6) is a pleiotropic cytokine with various cellular functions, among them the induction of acute phase proteins in the liver and the stimulation of B cells. On target cells, IL-6 initially binds an IL-6-specific α -receptor, the IL-6R. Upon binding of the surface-expressed IL-6R the IL-6/IL-6R complex leads to a homodimerization of gp130, the second subunit of the IL-6 receptor complex. A soluble form of the IL-6R (sIL-6R) can be generated via proteolytic cleavage or alternative splicing and the complex of IL-6 and sIL-6R is capable of activating cells which lack the membrane-bound IL-6R (IL-6-transsignaling). IL-6-transsignaling can be selectively inhibited by a naturally occurring, soluble form of gp130 (sgp130). Many inflammatory and neoplastic disorders have been attributed to sIL-6R mediated signaling and recombinant sgp130 is expected to be a valuable therapeutic tool to treat these diseases.

In this work transgenic mice were generated, which express sgp130Fc under a liver-specific promoter. Sgp130Fc is a fusion protein consisting of the extracellular part of gp130 and the Fc part of a human IgG antibody. Transgenic mice of the “first generation” showed comparably low sgp130Fc serum levels ($<0.55 \mu\text{g/ml}$), which were not sufficient to inhibit murine IL-6-transsignaling (in terms of acute phase induction by injection of Hyper-IL-6). New transgenic mice were created, which express sgp130Fc from a codon-optimized cDNA. In the “second generation” of sgp130Fc transgenic mice a more than 50-fold increase in sgp130Fc serum expression was achieved ($\leq 34.4 \mu\text{g/ml}$). It was shown that transgenic sgp130Fc protein is functionally active and that the amounts of sgp130Fc were sufficient to block murine IL-6-transsignaling. Northern blot analysis revealed sgp130Fc mRNA expression in liver, kidney, lung and muscle.

By employing the murine air pouch model of acute inflammation it was demonstrated that leukocyte accumulation in sgp130Fc transgenic mice was profoundly impaired compared to wildtype mice. In particular, neutrophil and macrophage infiltration was disturbed only at later stages of inflammation, which was paralleled by a reduced production of the CC chemokine MCP-1. High local levels of IL-6 were detected during the onset of inflammation, whereas sIL-6R peaked at later time points. By depleting the neutrophils at the site of inflammation it could be demonstrated that sIL-6R was released from transmigrating neutrophils. Taken together, these results indicate an important role for sIL-6R in mediating the transition between the initial, neutrophilic stage of infection and the later mononuclear cell influx, thereby promoting the resolution of inflammation.

6 Zusammenfassung

Interleukin-6 (IL-6) ist ein pleiotropes Zytokin und besitzt ein breites Wirkungsspektrum, das von der Induktion der Akut-Phase-Reaktion in der Leber bis zur Stimulation von B-Zellen zur Immunglobulinproduktion reicht. Auf Zielzellen bindet IL-6 zuerst an einen IL-6-spezifischen α -Rezeptor (IL-6R), woraufhin es zur Bindung und Dimerisierung der zweiten Rezeptoruntereinheit gp130 kommt. Neben dem membranständigen IL-6R existiert auch eine lösliche Form (sIL-6R), die entweder durch proteolytische Spaltung oder alternatives Spleißen entsteht. Der Komplex aus IL-6 und sIL-6R kann Zellen stimulieren, die lediglich gp130, aber keinen IL-6R tragen (IL-6-transsignaling). IL-6-transsignaling kann durch eine lösliche Form von gp130 (sgp130), die natürlicherweise im menschlichen Körper vorkommt, selektiv gehemmt werden. Viele Entzündungskrankheiten und Krebsformen beruhen auf IL-6-transsignaling und sgp130 wird in diesem Zusammenhang ein hohes therapeutisches Potential bei der Behandlung dieser Krankheiten zugesprochen.

Im Rahmen dieser Arbeit wurden transgene Mäuse generiert, die sgp130Fc unter der Kontrolle eines leberspezifischen Promotors exprimieren. Sgp130Fc ist ein Fusionsprotein, das aus dem extrazellulären Teil von gp130 und dem Fc-Teil eines humanen IgG-Antikörpers besteht. In transgenen Mäusen der „ersten Generation“ wurden vergleichsweise geringe Mengen von sgp130Fc im Serum gefunden ($<0.55 \mu\text{g/ml}$), welche nicht ausreichten, um murines IL-6-transsignaling zu inhibieren (Induktion der Akut-Phase-Antwort nach Hyper-IL-6-Injektion). Daraufhin wurden neue transgene Mäuse generiert, die sgp130Fc nun von einer codon-optimierten cDNA exprimierten. Die Tiere der „zweiten Generation“ wiesen einen mehr als 50-fachen Anstieg der Konzentration von sgp130Fc im Serum auf ($\leq 34.4 \mu\text{g/ml}$), der ausreichend war, um IL-6-transsignaling in der Maus zu hemmen. Im Northern blot konnte gezeigt werden, daß sgp130Fc in der Leber, Niere, Lunge und im Muskel der transgenen Mäusen exprimiert wird.

In einem Krankheitsmodell, bei dem künstlich eine akute Entzündungsreaktion in der Maus hervorgerufen wird (Air-pouch-Modell), konnte nachgewiesen werden, daß die Akkumulation von Leukozyten am Ort der Entzündung in sgp130Fc-transgenen Mäusen stark beeinträchtigt ist. Es stellte sich heraus, daß insbesondere die Akkumulation von Neutrophilen und Makrophagen im späteren Entzündungsverlauf gestört ist. Dies ging mit einer reduzierten Produktion des Chemokins MCP-1 am Entzündungsherd einher. Zu Beginn der Entzündung wurden hohe Konzentrationen von IL-6 nachgewiesen, wohingegen die Menge an löslichem IL-6R erst im späteren Verlauf der Entzündung anstieg. Weiterhin konnte mit Hilfe von

neutropenischen Mäusen gezeigt werden, daß der lösliche IL-6R von Neutrophilen stammt, die in den Entzündungsherd eingewandert sind. Die Ergebnisse dieser Arbeit deuten daraufhin, daß das IL-6-transsignaling den Übergang von der anfänglichen, von Neutrophilen dominierten Phase zur späteren, mononukleären Phase einer akuten Entzündung vermittelt. Somit spielt der lösliche IL-6R eine entscheidende Rolle bei der erfolgreichen Auflösung einer akuten entzündlichen Reaktion.

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8 Appendix

8.1 Sequences

A Nucleotide and amino acid sequence of the transgene expression cassette pEPCK-sgp130Fc

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51  CCCCTGATTC  TGTGGATAAC  CGTATTNCCG  CCTTTGAGTG  AGCTGATACC
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151 GGAAGAGCGC  CCAATACGCA  AACCGCCTCT  CCCC GCGCGT  TGGCCGATTC
201 ATTAATGCAG  CTGGCACGAC  AGGTTTCCCG  ACTGGAAAGC  GGCAGTGAG
251 CGCAACGCAA  TTAATGTGAG  TTAGCTCACT  CATTAGGCAC  CCCAGGCTTT
301 ACACTTTATG  CTTCCGGCTC  GTATGTTGTG  TGGAATTGTG  AGCGGATAAC
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                               BamHI
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551 AGAGGCGTCC  CGGCCAGCCC  TGTCCCTGAC  CCCCACCTGA  CAATTAAGGC
601 AAGAGCCTAT  AGTTTGCATC  AGCAACAGTC  ACGGTCAAAG  TTTAGTCAAT
651 CAAACGTTGT  GTAAGGACTC  AACTATGGCT  GACACGGGGG  CCTGAGGCCT
701 CCCAACATTC  ATTAACAACA  GCAAGTTCAA  TCATTATCTC  CCCAAAGTTT

                               NcoI
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901 AGCACGGTTG  GCCTTCCCTC  TGGGAACACA  CCCTTGGCCA  ACAGGGGAAA

                               XbaI
951 TCCGGCGAGA  CGCTCTGAGA  TCCTCTAGAC  CCCGCAAGAT  GTTGACGTTG
                               M  L  T  L
1001 CAGACTTGGG  TAGTGCAAGC  CTTGTTTATT  TTCCTCACCA  CTGAATCTAC
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XbaI

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XhoI

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BamHI

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EarI
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XhoI
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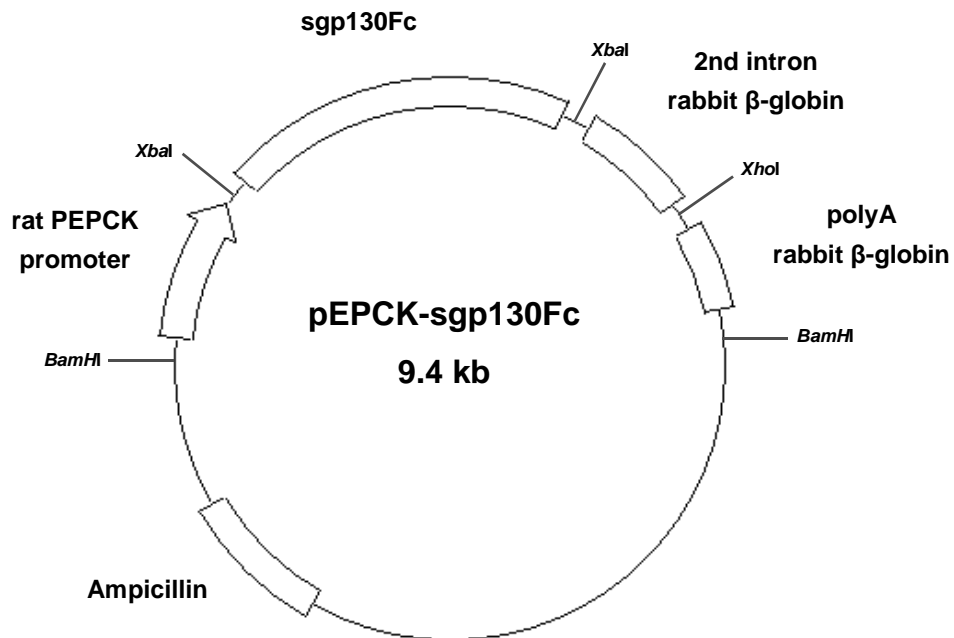
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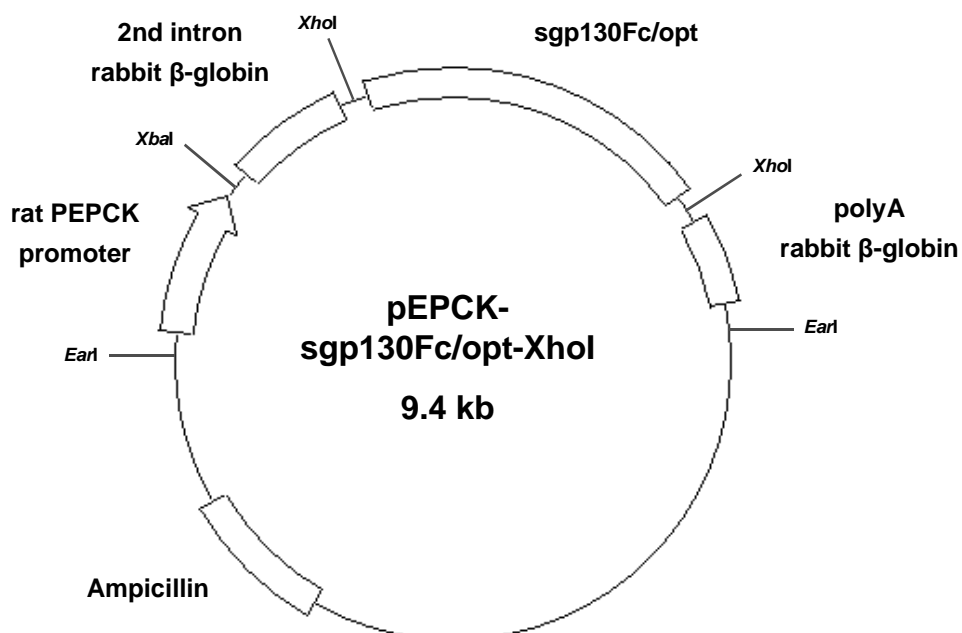
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8.2 Vector maps

A pEPCK-sgp130Fc



B pEPCK-sgp130Fc/opt-XhoI



8.3 Abbreviations

ADAM - a disintegrin and metalloprotease

Ag – antigen

Amp – ampicillin

AP – alkaline phosphatase

APC - allophycocyanin

APP – acute phase protein

APS – ammonium persulfate

ATCC - American Type Culture Collection

Bcl-2 - B-cell lymphoma 2

BSA – bovine serum albumine

CBM - cytokine-binding module

CD – cluster of differentiation

cDNA – complementary DNA

CIAP - calf intestine alkaline phosphatase

CLC - cardiotrophin-like cytokine

CNTF - ciliary neurotrophic factor

CT-1 – cardiotrophin-1

dATP - deoxy adenosine triphosphate

DEAE – diethylaminoethyl

DNA - deoxyribonucleic acid

DMSO – dimethylsulfoxide

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme linked immunosorbent assay

ER – endoplasmic reticulum

FACS - fluorescence-activated cell-sorting

FCS – fetal calf serum

Fig - figure

FITC – fluorescein isothiocyanate

FRET - fluorescence resonance energy transfer

GFP – green fluorescent protein

gp – glycoprotein

Grb2 - growth-factor-receptor-bound protein

Gro α - growth-related oncogene α
h - hour
HHV-8 – human herpesvirus-8
H-IL-6 – Hyperinterleukin-6
his – histidin
hIL-6 – human interleukin-6
ICAM-1 - intercellular adhesion molecule-1
IFN α , γ – interferon α , γ
IgG – immunoglobulin G
IL – interleukin
IP - immunoprecipitation
JAK – janus-kinase
kb – kilobase pairs
kD – kilodalton
kg - kilogram
KS – Kaposi's sarcoma
KSHV - Kaposi's sarcoma associated herpesvirus
l – liter
LB - lysogeny broth
LIF - leukemia inhibitory factor
LPS - lipopolysaccharide
MCP-1/3 - Monocyte chemoattractant protein-1/3
mAB – monoclonal antibody
MAPK - mitogen activated protein kinase
MCD - multicentric Castleman's disease
mg – milligram
 μ g - microgram
min - minute
MM – multiple myeloma
ml - milliliter
Mr – molecular weight
mRNA – messenger RNA
ng - nanogramm
NF – nuclear factor

NPN - new neuropoietin
OD – optic density
o/n – over night
ORF – open reading frame
OSM – oncostatin M
PBS - phosphate buffered saline
PCR – polymerase chain reaction
PEL - primary effusion lymphoma
PEPCK - phosphoenolpyruvate-carboxykinase
pNPP – 4-nitrophenyl phosphate
POD - peroxidase
P-STAT3 - phospho-STAT3
RA - rheumatoid arthritis
RNA - ribonucleic acid
rpm – round per minute
RT – room temperature
RT-PCR - reverse transcription polymerase chain reaction
SAA – serum amyloid A
SD - standard deviation
SDS – sodium dodecyl sulfate
SDS-PAGE - sodium dodecyl sulphate – polyacrylamide gel electrophoresis
sec - second
sgp130 – soluble glycoprotein 130
sIL-6R - soluble interleukin-6 receptor
SOCS – suppressor of cytokine signaling
ssDNA – single stranded DNA
STAT - signal transducer and activator of transcription
TACE - TNF α converting enzyme
TBS – tris buffered saline
TEMED – N,N,N',N'-tetraethylenamine
tg - transgenic
TGF β - transforming growth factor β
T_m – melting temperature
TNF α – tumor necrosis factor α

UV - ultraviolet

VCAM-1 - vascular cell adhesion molecule-1

vIL-6 – viral interleukin-6

WT - wildtype

8.4 Publications

Scheller, J., Kovaleva, M., **Rabe, B.**, Eichler, J., Kallen, K.-J. and S. Rose-John. Development of a monoclonal antibody-based enzyme-linked immunoabsorbent assay for the binding of gp130 to the IL-6/IL-6R complex and its competitive inhibition, *Journal of Immunological Methods*, 2004, 291(1-2):93-100

Kovaleva, M., Bussmeyer, I., **Rabe, B.**, Grötzinger, J., Sudarman, E., Eichler, J., Conrad, U., Rose-John, S. and J. Scheller. Abrogation of viral interleukin-6 (vIL-6)-induced signaling by intracellular retention and neutralization of vIL-6 with an anti-vIL-6 single-chain antibody selected by phage display, *Journal of Virology*, 2006, 80(17):8510-20

Chalaris, A., **Rabe, B.**, Paliga, K., Lange, H., Laskay, T., Fielding, C.A., Jones, S.A., Rose-John, S. and J. Scheller. Apoptosis is a natural stimulus of IL6R shedding and contributes to the pro-inflammatory trans-signaling function of neutrophils, 2007, submitted

Rabe, B., Chalaris, A., Seegert, D., Rose-John, S. and J. Scheller. Blocking IL-6-transsignaling *in vivo* effects in abrogation of inflammation, 2007, in preparation

8.5 Curriculum vitae

Name: Björn Rabe
Geburtstag: 20.4.1973
Geburtsort: Kiel
Staatsangehörigkeit: deutsch

2003 – 2007 Anfertigung der vorliegenden Arbeit in der Arbeitsgruppe Prof. Dr. Rose-John am Biochemischen Institut der Christian-Albrechts-Universität zu Kiel

2002 – 2003 Diplomarbeit am Institut für Mikrobiologie der Bundesforschungsanstalt für Ernährung und Lebensmittel (Standort Kiel): „ Molekulare Charakterisierung von Deletionsderivaten des *S. thermophilus* Bakteriophagen TP-J34“

1994 - 2002 Studium der Biologie an der Christian-Albrechts-Universität zu Kiel (Hauptfach: Mikrobiologie; Nebenfächer: Zellbiologie, Biochemie)

1993 – 1994 Studium der Medizin an der Christian-Albrechts-Universität zu Kiel

1992 – 1993 Zivildienst beim Malteser-Hilfsdienst Kiel, Rettungssanitäter

1983 – 1992 Ernst-Barlach-Gymnasium Kiel, Abitur 1992

1979 – 1983 Grundschule Suchsdorf, Kiel

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Eidesstattliche Erklärung

Hiermit versichere ich, Björn Rabe, 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 21.3.2007

Björn Rabe