Characterization of inflammatory signaling
in the tumor microenvironment
using SOCS3-U mice

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The true delight is in the finding out rather than in the knowing.

- Isaac Asimov
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<td>AAV</td>
<td>adeno associated virus</td>
</tr>
<tr>
<td>Alb</td>
<td>Albumin</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethane</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CDS</td>
<td>coding sequence</td>
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<tr>
<td>cf.</td>
<td>confer</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>Cre</td>
<td>causes recombination (recombination enzyme)</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DEN</td>
<td>DiethylNitrosamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sulfate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ES cells</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>F</td>
<td>Farad</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FLP</td>
<td>flippase (recombination enzyme)</td>
</tr>
<tr>
<td>FRT</td>
<td>FLP recognition target</td>
</tr>
<tr>
<td>(e)GFP</td>
<td>(enhanced) green fluorescent protein</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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</table>
IRES  
internal ribosomal entry site

ITR  
inverted terminal repeat

kb  
kilo bases

kD  
kilo Dalton

KO  
knock out

LAH  
left arm of homology

LB  
lysogeny broth

LIF  
leukemia inhibitory factor

loxP  
locus of X-over P1

LPS  
Lipopolysaccharide

M  
molar

MEF  
mouse embryonic fibroblast

MMC  
Mitomycin C

mRNA  
messenger ribonucleic acid

NaOH  
Sodium hydroxide

NaCl  
Sodium chloride

Na$_3$C$_6$H$_5$O$_7$  
Sodium citrate

Na$_3$VO$_4$  
Sodium orthovanadate

ORF  
open reading frame

PAGE  
Polyacrylamide gel electrophoresis

PBS  
phosphate buffered saline

PCR  
polymerase chain reaction

PMA  
Phorbol 12-Myristate 13-Acetate

PMSF  
Phenylmethanesulfonylfluoride

PVDF  
Polyvinylidendifluorid

qPCR  
quantitative polymerase chain reaction

rAAV  
recombinant adeno associated virus

RAH  
right arm of homology

RNA  
ribonucleic acid

rox  
region of cross-over

rpm  
rounds per minute

RT  
room temperature

ssDNA/RNA  
single stranded DNA/RNA

SDS  
sodium dodecyl sulfate

SEM  
standard error of the mean

SOCS3  
suppressor of cytokine signaling 3

STAT3  
signal transducer and activator of transcription 3

Sv  
sievert

TBS  
tris buffered saline

TBS-T  
tris buffered saline plus 0.1% Tween-20

TE  
Tris-HCl EDTA
<table>
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<th>Abbreviation</th>
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<tr>
<td>TG</td>
<td>transgene</td>
</tr>
<tr>
<td>Tk</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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Abstract

Inflammatory signaling in the tumor microenvironment has been increasingly recognized to be a common, critical driving force for cancerogenesis. Unraveling inflammatory signaling in cancer initiation might give further insights into cancer formation to develop novel therapeutic treatment possibilities. This study introduces SOCS3-U, a novel reporter mouse line to conditionally visualize inflammatory signaling. The SOCS3-U modification was targeted to the endogenous suppressor of cytokine signaling 3 (SOCS3) locus, a negative feedback regulator of the JAK/STAT pathway highly upregulated by a variety of key inflammatory mediators. Here, a loxP flanked stop cassette in the first intron of SOCS3-U prevents the compound expression of SOCS3 and IRES-GFP. Thus, Cre-mediated recombination of the loxP flanked stop cassette leads to GFP expression in those cells that have upregulated SOCS3 expression. Moreover, the SOCS3 ORF has been flanked by rox sites to generate conditional SOCS3 knock-out mice in a Dre/rox-dependent manner, and the GFP configuration of SOCS3-U can be switched to firefly luciferase. In first experiments, SOCS3-U mice were used in mouse models of chemically induced colorectal and hepatocellular carcinoma to identify activation of distinct immune cell populations. These experiments demonstrate that SOCS3 negative M2 macrophages specifically upregulate CCL20 that attracts CCR6 expressing lymphocytes to the inflamed colon as a driving force for colorectal cancerogenesis. In the liver, inflammation promotes the formation of a hitherto unknown NK-T cell subpopulation that specifically upregulates IL-6Ra. Moreover, a novel generated AlbDre BAC transgenic mouse line creates hepatocyte specific SOCS3 knock-out mice via the Dre/rox system, providing the opportunity for sophisticated combinatory mouse models using more than one recombinase. Taken together, this study provides a
novel genetic tool for the universal visualization of inflammatory signaling in vivo.
für elegante Mausmodelle mit mehr als einer Rekombinase ermöglicht. Zum-
mengenommen stellt diese Arbeit ein neues, genetisches Werkzeug zur universellen
Identifizierung von Entzündungsreaktionen in vivo bereit.
1 Introduction

The development of a complex, higher organism from a single, fertilized oocyte is a vastly complicated process, and the evolution to multicellular organisms justifiably took place over the course of billions of years.

1.1 Inflammation associated cancerogenesis

Once an organism is fully developed, tissue growth is restricted to very defined areas harboring adult stem cells. Aberrations from this very controlled tissue growth are called neoplasms, which are further divided into benign neoplasms, whose growth does not spread -or metastasize- to neighboring tissues, and malignant neoplasms, which have the potential to metastasize to other tissues. Malignant neoplasia -or cancer- therefore does not constitute a singular disease, but rather encompasses uncontrolled cell growth in almost every tissue and invasion of these malignant cells into other tissues. The various cancer types display a high variability in incidence, mortality, severity, associated risk factors or geographical distribution (Fig. 1.1). There are, however, certain common hallmarks shared by all cancer types [seminally reviewed by Hanahan & Weinberg, 2011]. Cancer has been a constant burden on human health throughout history, with the earliest references preserved dating back to 3,000 BC [Hajdu, 2011].

In 2012, estimated 8.2 million cancer related deaths for the first time outpaced coronary heart diseases (7.3 million) and stroke (6.6 million), the undisputed leaders of the cardiovascular diseases (CVDs) and primary cause of death (17.5 million combined for CVDs), with 14.1 million new cancer cases world-wide [WHO, GLOBOCAN 2012, by Ferlay et al., 2015]. Among cancers, colorectal cancer has the
third highest incidence (1.4 million new cases) with 694,000 deaths (mortality to incidence ratio of 0.49) in 2012, with a higher occurrence of about 55% in developed regions. Liver cancer ranks only fifth in incidence (782,000 new cases) but has a staggering mortality to incidence ratio of 0.95 (745,000 deaths), with higher occurrence in males and a strong bias towards less developed regions (83% of new cases). Most cancer types have a rather late onset and further deteriorate over time, and so the relative cancer occurrence is higher in developed regions; a trend which is likely to continue, given the increasing age-average of populations in developed regions. Consistently, European regions account for roughly 25% of cancer incidence and 20% of cancer deaths but accommodate only 10% of the global population.

1.1.1 Influence of the tumor microenvironment on cancerogenesis

In the last decades, our paradigm how we define cancer has substantially changed. Cancer is not anymore viewed as a few of out-of-control cells, but rather a network of different cell types surrounding and permeating masses of proliferating cells, almost forming tissue of their own. In fact, it is now believed that the interactions of cells of this so called tumor microenvironment with each other and the tumor cells are enabling cancerogenesis in the first place, a notion that becomes more apparent if the hallmarks of cancer are considered in more detail. Even if our conception of
tumors has broadened, the ability to proliferate and replicate indefinitely remains to be the prime hallmark of cancer cells. This includes a susceptibility to growth factors released by cancer cells themselves or immune cells in the tumor microenvironment, but also the ability to circumvent negative proliferation regulators, like contact inhibition. The classical countermeasure to a over-proliferating cell is for the cell to undergo apoptosis, either by cell-intrinsic mechanisms, or by ligand-induced, extrinsic apoptosis. A developing tumor therefore has to be able to both suppress the intrinsic apoptotic machinery, as well as to evade extrinsic apoptosis. The heightened replicative and proliferative potential of tumor cells requires a continuous supply of nutrients and oxygen, which the tumor achieves by inducing enhanced vascularization. The last step in tumorigenesis is the invasion and metastasis of hitherto unaffected tissues, which can also be facilitated by non-tumor cells within the microenvironment [Gocheva et al., 2010].

Immune cells infiltrate the tumor microenvironment in high numbers and inhere a critical role in tumorigenesis, albeit a dichotomous one. Developing tumors need to escape destruction by immune cells, as becomes apparent if the increase in cancer formation upon immunodeficiency is considered [Vajdic & van Leeuwen, 2009]. Continuous inflammation however has tumor promoting functions, in that immune cells can release reactive oxygen species which can induce DNA damage in cancer cells, contributing to the genomic instability and mutagenesis [Maeda & Akaike, 1998]. Additionally, immune cells secrete various cytokines and chemokines into the tumor microenvironment, some of which, e.g. EGF (epidermal growth factor), VEGF (vascular endothelial growth factor) or FGF2 (basic fibroblast growth factor), actively induce proliferation. This is especially true for cells of the innate immune system, which is heavily involved in wound healing and tissue remodeling. Consequently, tumors have been long compared to „wounds that do not heal” [Dvorak, 1986].

Very similar to the inflammatory response to a wound, a developing tumor attracts and activates leukocytes to the afflicted tissue [Mantovani et al., 2004].
Amongst the first leukocytes recruited are neutrophils, followed by monocytes differentiating into macrophages. These macrophages will then strongly secrete growth factors and cytokines into the tumor microenvironment, impacting on the endothelial, epithelial and mesenchymal cells in the vicinity [Coussens & Werb, 2002]. Additional cytokines and chemokines are secreted from the tumor cells, e.g. IL-6 (interleukin-6) and CSF-1 (colony stimulating factor 1), which push the differentiation of myeloid cells to tumor associated macrophages (TAMs) [Allavena et al., 2000]. Although TAMs kill neoplastic cells via phagocytosis to a certain extent upon IL-2 or IL-12 stimulation, they also release angiogenic and lymphangiogenic factors that stimulate neoplasia [Schoppmann et al., 2002]. The tumor microenvironment is generally recognized as having high levels of IL-10, blocking cytotoxic T-lymphocytes, and TGF-β (transforming growth factor beta), while having low levels of IL-12 and a defective T\(_{H1}\) response [Germano et al., 2008]. The low levels of the anti-carcinogenic IL-12 coincides with an increase in pro-carcinogenic IL-23, mediated by STAT3 (signal transducer and activator of transcription 3) [Kortylewski et al., 2009]. Likewise, TAMs have a strong bias towards the M2 polarization [Mantovani et al., 2002], skewed by the survival-factor NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) [Hagemann et al., 2008].

1.1.2 Hepatic inflammation drives hepatocellular carcinogenesis

The vast majority (85%-90%) of liver cancer is represented by hepatocellular carcinoma (HCC) [El-Serag, 2011], which is mainly driven by cirrhosis after hepatitis B (HBV infection) or C (HCV infection), prolonged alcohol consumption or non-alcoholic steatohepatitis (NASH) [Donato et al., 2002; Yoshioka et al., 2004; Sherman, 2005]. Common hallmark of cirrhosis is an enhanced inflammation, and elevated serum levels of IL-6 can be found in patients with HBV, HCV or sustained alcohol consumption [Khoruts et al., 1991; Kakumu et al., 1991; Malaguarnera et al., 1997], whereas obesity generally is increasingly recognized as a state of chronic low grade inflammation [Hotamisligil et al., 1993]. Persistent inflammatory signal-
ing in the liver leads to wide-spread hepatocyte death, which is compensated by increased proliferation, further contributing to carcinogenesis [Bisgaard & Thorgeirsson, 1996]. NF-κB, mediating the effect of proliferation on the balance of proliferation and apoptosis, has consequently been recognized as a tumor promoter in inflammation-associated cancerogenesis [Pikarsky et al. 2004]. Consistently, high levels of the catalytic subunits IKKα (IκB kinase α) and IKKβ are a prerequisite for malignancy in hepatocellular carcinoma [Jiang et al. 2010].

Hepatocyte specific deletion of IKKβ increases chemically-induced, via injection of the carcinogen diethylnitrosamine (DEN), HCC development, possibly due to enhanced cell death due to an elevated JNK (c-Jun N-terminal kinase) activity and concomitant ROS (reactive oxygen species) accumulation, consistently accompanied by increased hepatocyte proliferation [Maeda et al. 2005]. Interestingly, IKKβ ablation both in hepatocytes and Kupffer cells (KCs) has the opposite effect and decreases DEN-induced HCC susceptibility, while exhibiting higher levels of liver injury and cell death and no detectable IL-6 upon DEN injection. Kupffer cells, liver resident macrophages, are important inflammatory contributors and the major source for proliferation stimulating mitogens in the liver [Fausto 2000]. Increasing levels of IL-1α, released from dying hepatocytes, induces IL-6 release from KCs, elevating the inflammatory tone and subsequently causing even more hepatocyte death and resulting hyperproliferation, a potential driving force for HCC development [Naugler et al. 2007; Sakurai et al. 2008]. Compellingly, this effect is more pronounced in males, mirroring the enhanced risk for men to develop HCC, whereas the IL-6 release by KCs in females is inhibited by high levels of estrogen [Mantovani 2007]. In line with the tumor promoting effect of a hepatocyte specific IKKβ deletion, loss of the NF-κB regulatory subunit IKKγ in hepatocytes causes steatohepatitis and spontaneous HCC development [Luedde et al. 2007]. Additionally, hepatocyte specific deletion of the TLR (toll-like receptor) signaling mediator MyD88 (myeloid differentiation primary response gene 88) reduces DEN-induced HCC formation via decreased activation of both NF-κB and JNK pathways [Ströhle,]
Obesity is a major risk factor for hepatocellular carcinoma

Obesity has been recognized as a state of chronic low grade inflammation and increases the risk of almost all cancers, with varying severity for individual cancer types per gender [Calle et al., 2003]. People with a body mass index (BMI, \( \frac{\text{weight}[kg]}{\text{height}^2[m]} \)) of at least 40 had dramatically increased death rates for all cancers combined (52% for men and 62% for women respectively) compared to men and women with normal weight (BMI between 18.5 and 24.9). Amongst men, a BMI of at least 35 increases the relative risk to die from liver cancer 4.52 fold, the strongest increase in relative risk of death for all cancers (Fig. 1.2). As a consequence, an estimated 90,000 death per year in the USA are attributed to a BMI greater than 25.

In line with the obesity-associated increased risk of developing liver cancer, high-fat diet (HFD) fed rats develop not only NASH but have a higher incidence of pre-cancerous markers and pre-neoplastic lesions upon DEN injection [Wang et al., 2009]. Consistently, HFD feeding aggravates spontaneous HCC development upon
hepatocyte specific IKKγ deletion in mice [Wunderlich et al., 2008]. NASH development as a cause for HCC is furthermore dependent on the non-canonical NF-κB pathway, as LIGHT (TNFsF14) secretion from NK-T cells promotes hepatic lipid uptake and ultimately leads to cancer formation [Wolf et al., 2014]. The exacerbated tumorigenesis accompanying obesity is reversed in a whole body IL-6 knock-out, in line with the previously demonstrated role for IL-6 in DEN-induced HCC formation [Park et al., 2010]. Interestingly, in contrast to the IL-6 deletion, whole body IL-6Ra deletion fails to exert a protective effect on obesity promoting HCC development [Gruber et al., 2013]. On normal chow diet (NCD), IL-6 signaling inhibits GSK-3β, a serine/threonine protein kinase targeting the key anti-tumor regulator Mcl-1 for polyubiquitinylation by MULE (Mcl-1 ubiquitin ligase E3), thereby prohibiting hepatocyte apoptosis and promoting cancerogenesis. In concert with decreased GSK-3β activity upon IL-6 signaling, expression of MULE and PP-1α, a GSK-3β activating protein phosphatase, is inhibited in a pSTAT3 dependent manner. Loss of IL-6Ra therefore protects from DEN-induced HCC formation by destabilizing Mcl-1, subsequently increasing apoptosis of damaged hepatocytes. Obesity aggravates tumor formation in the liver by further stabilizing Mcl-1 in an IL-6 independent manner, enabling continued proliferation of malformed hepatocytes.

Intriguingly, a hepatocyte specific IL-6Ra deletion does not confer protection from DEN-induced HCC development even on NCD, whereas T-cell specific IL-6Ra ablation (IL-6Ra T-KO) protects from DEN-induced HCC even under obese conditions [Gruber, 2013]. The function of effector T-cells is tightly controlled by regulatory T-cells (TregS), and combined IL-6 and IL-1 signaling is required to release effector T-cells from Treg-mediated repression and mount both a strong T H1 and T H17 response [Nish et al., 2014; Schenten et al., 2014]. Consequently, Treg depletion via i.p. injection of an α-CD25 antibody in the tumor initiation phase completely abrogates the protective effect of IL-6Ra T-KO on DEN-induced HCC development [Gruber, 2013].
1.1.3 Inflammatory bowel disease and gut microbiota

Although increased inflammation in the tumor microenvironment is a characteristic of every cancer type, some cancers show an especially high association of chronic inflammation with cancerogenesis. Colorectal cancer (CRC) develops predominantly after continuous inflammatory bowel disease, e.g. chronic ulcerative colitis or Crohn’s disease [Jess et al., 2005; Danese et al., 2011]. Development of CRC is recapitulated in a mouse model of colitis associated cancerogenesis (CAC), where mice are injected with the procarcinogen azoxymethane (AOM) and tumorigenesis is promoted by induction of colitis with dextran sulfate sodium salt (DSS) application [Tanaka et al., 2003]. The enhanced inflammatory profile upon colitis exerts a deleterious effect on tumorigenesis, as becomes apparent in light of an enterocyte specific IKKβ deletion. Loss of IKKβ and therefore canonical NF-κB ablation dramatically reduces AOM/DSS induced tumor formation by 75% [Greten et al., 2004]. While the inflammatory tone is unaffected, IKKβ is required for Bcl-XL (B-cell lymphoma-extra large) expression, thus IKKβ ablation protects from CRC development via enhanced apoptosis. Interestingly, IKKβ deletion in the myeloid lineage also protects from AOM/DSS induced tumor formation, albeit to a smaller extent, but does so by reducing the inflammatory tone.

Inflammation in the colon is furthermore affected by the gut microbiome, which is in constant contact with the colonic mucosa, the tissues with the highest proliferative capacity, completely renewing the single-cell epithelial layer every 4-5 days [van der Flier & Clevers, 2009]. The gut microbiome in the large intestine contains over 10^{11} cells per g content and can differ greatly between individuals, further influenced by the dietary composition [Louis et al., 2014]. Metabolites produced by gut microbiota can have both protective and deteriorating effects, highlighting the importance of a healthy gut microbiome. Short-chain fatty acids (SCFAs), like butyrate and propionate, can inhibit histone deacetylases in colonocytes and immune cells, affecting signal transduction via transcription factors, ultimately leading to a downregulation of pro-inflammatory cytokines, like IL-6 and IL-12, and thereby
counteracting tumorigenesis [Chang et al., 2014]. Additionally, SCFAs are implicated in driving the differentiation of FoxP3+ (forkhead box P3) T_{reg}s, which exert an inflammation controlling effect [Smith et al., 2013]. Furthermore, butyrate signaling can inhibit NF-κB activation and selectively drive apoptosis in malformed cells [Thangaraju et al., 2009].

Conflicting data however show a tumor-promoting effect of butyrate in a mouse model of mutations both in the Apc (adenomatous polyposis coli) and Msh2 (MutS homolog 2) genes [Belcheva et al., 2014]. Other detrimental metabolites, like hydrogen sulfite or bile acid, on the other hand, drive the expression of pro-inflammatory cytokines and can cause DNA damage in colonocytes [Roediger et al., 1997; Islam et al., 2011]. Elevated levels of these pathogenic microbiota will eventually lead to their detection by dendritic cells via microorganism-associated pattern (MAMPs), subsequently promoting a T_{H17} mediated IL-23 upregulation, concomitantly down-regulating IL-10 [Grivennikov et al., 2012]. This continuing inflammatory signaling, caused by pathogenic bacteria, can lead to loss of colonocyte barrier function, enabling the efflux of gut microbiota from the colon lumen into the submucosa, ultimately further driving inflammatory signaling.

The diverse effects of the various microbiota, often dependent on a very fine-tuned balance between tumor-promoting and -inhibiting bacteria, sometimes even controversial effects of the same metabolite (e.g. butyrate), place the gut microbiota at a very delicate position in tumorigenesis, substantiated by microbiota affecting the efficacy of immunotherapy against other cancer entities [Garrett, 2015].

1.2 Supressor of cytokine signaling 3 (SOCS3)

Inflammatory signaling, whether it being pro- or anti-inflammatory, is a very important tool for an organism to elicit a strong, specific and rapid response to a particular stimulus, and it is therefore paramount to tightly control both its onset as well as termination. Cytokines are potent mediators of inflammatory signaling and are a cornerstone of the immune response, but can also control proliferation or
contribute to tissue remodeling. Cytokines are usually kept in low concentrations but can be released in high quantities in a very short amount of time, so that their effect on their respective target tissues runs at peak efficiency. The intracellular effect of cytokine signaling can have a profound impact on various other signaling cascades as well as dramatically change gene expression, so unchecked inflammatory signaling can very well exceed the deleterious effects of its cause, and is in some cases fundamental part of the problem in the first place. Eukaryotic cells have therefore a wide array of ways to terminate inflammatory signaling at their disposal, one of it being the SOCS family of eight proteins.

1.2.1 SOCS mediated cytokine signaling feedback inhibition

The SOCS family of proteins comprises eight proteins, SOCS1-7 and CIS (cytokine-inducible Src-homology 2-containing protein). The eponymic SOCS1 was first described in parallel by three groups in 1997 [Starr et al.; Naka et al.; Endo et al.], and CIS in 1995 [Yoshimura et al.]. The shared functional significance of the SOCS protein family members as negative feedback inhibitors is reflected in their structural similarity (Fig. 1.3), containing a central SH2 (Src homology 2) domain and a highly conserved SOCS box at the C-terminus [Hilton et al. 1998]. SOCS1 and SOCS3 contain an additional, conserved kinase inhibitory region (KIR domain) at their N-terminus [Yasukawa et al. 1999]. The respective domains dictate three potential routes of dissipating inflammatory signaling for SOCS proteins.

**Mechanism of SOCS3 negative feedback regulation**

The most prominent domain is the C-terminal SOCS box, which can interact with the elongin B/C heterodimer and cullin-5 to form an E3 ubiquitin ligase [Zhang et al. 1999, Zheng et al. 2002]. SOCS proteins can utilize this domain to directly target cytokine receptors, JAKs (janus kinases) or downstream mediators of inflammatory signaling for degradation [Kamura et al. 2004]. Although there are certain targets for SOCS3 E3 ligase activity known [Williams & Palmer 2012], the affinity
of SOCS3 for cullin-5, and thereby ultimately its E3 ligase activity, is comparably low [Babon et al., 2009], indicating that SOCS3 is inhibiting inflammatory signaling rather through its SH2 or KIR domains than an E3 ligase activity via its SOCS box. SOCS proteins are inhibitors of cytokine signaling through the JAK/STAT pathway and are able to rapidly decrease activated STAT transcription factors. IL-6 stimulation leads to phosphorylated STAT3 (pSTAT3) in hepatocytes with one or two copies of SOCS3 within 15 minutes and persists for 2h, which is prolonged to 4h in the absence of SOCS3 [Croker et al., 2003]. SOCS proteins bind with their SH2 domain to phosphotyrosines on various cytokine receptors, e.g. pY757 on gp130 (glycoprotein 130), pY800 on IL-12Rβ2 (IL-12 receptor β2) or pY985 on the leptin receptor (LepR) in case of SOCS3, with a much higher affinity than JAKs, thereby competitively inhibiting binding and subsequent activating phosphorylation of JAKs [Nicholson et al., 2000]. SOCS proteins with a KIR domain, like SOCS3, can also directly bind and inhibit JAKs (Fig. 1.4). In fact, SOCS3 specifically binds receptor/JAK dimers, which, together with the selective affinity for certain receptors, may explain why SOCS3, although being able to bind to three of the four JAK/STAT kinases (JAK1, JAK2 and TYK2, but not JAK3), does not inhibit signal-
**Figure 1.4: Mechanism of SOCS3 feedback inhibition on JAK/STAT signaling**

Binding of the ligand induces dimerization and subsequent activation of associated JAKs. JAK phosphorylation of the respective receptor β-chains triggers recruitment and phosphorylation of downstream STAT signaling mediators. Phosphorylated STAT dimers translocate to the nucleus and activate target gene expression, including SOCS3. SOCS3 translocates to the plasma membrane and inhibits JAK/STAT signaling. [from Babon & Nicola, 2012]

SOCS3 expression is induced by various stimuli

SOCS3 expression is mainly induced by signaling through the JAK/STAT pathway, but is upregulated differentially in response to the respective upstream mediator of JAK/STAT signaling. *i.v.* injection of IL-6 results in an upregulation of SOCS3 mRNA in mouse livers within 20 minutes and returns to basal levels after 8 h [Starr et al., 1997]. The SOCS3 promoter contains two STAT1/STAT3 responsive elements [Auernhammer et al., 1999] and is most prominently induced by IL-6 type cytokines. Apart from IL-6, IL-11 and LIF as IL-6 type cytokines, SOCS3 is also upregulated by leptin [Bjørbaek et al., 1998], TNFα [Emanuelli et al., 2001], CNF [Bjørbaek et al., 1999], insulin [Emanuelli et al., 2000] and resistin [Steppan et al., 2005]. Expression of SOCS3 is also activated by IL-10 derived pSTAT3, but SOCS3 does not inhibit signaling by IL-10, a cytokine with classical anti-inflammatory capacities [Yasukawa et al., 2003; Lang et al., 2003; Niemand et al., 2003].
SOCS3 activity is efficiently terminated by post-translational modification

The efficient inhibition of JAK/STAT signaling by SOCS3 requires an equally efficient termination of that inhibition, in order to normalize the transcriptome of the target cell and prime it for an upcoming, novel signaling event. Whereas JAK/STAT inhibition by SOCS3 will prevent further de novo expression, SOCS3 is post-translationally regulated through several mechanisms. The SOCS3 SH2 domain contains an unstructured region containing a PEST sequence [Babon et al., 2005], a motif commonly associated with a short protein half-life [Rogers et al., 1986]. Furthermore, SOCS3 is tyrosine phosphorylated at residues Y204 and Y221 in the SOCS box, inhibiting the interaction of SOCS3 with elongin C and targeting SOCS3 for proteasome-mediated degradation [Haan et al., 2003]. Proteasomal degradation of SOCS3 can also be achieved via polyubiquitination at SOCS3 K6, a conserved residue absent in a naturally occurring truncated form of SOCS3 via alternative translation after ER stress or activated protein kinase (PKR) [Sasaki et al., 2003].

Furthermore, SOCS3 is regulated by promoter methylation and miRNA action [Boosani & Agrawal, 2015]. miR-122, the most abundant miRNA in the liver, is a potent regulator of SOCS3 activity, although the mechanism is not completely elucidated. Conflicting reports in Huh7 HCC cells show either an increased SOCS3 promoter methylation in the absence of miR-122, resulting in silencing of SOCS3 expression and subsequently enhanced phosphorylation of STAT3 upon interferon-α (IFN-α) stimulation [Yoshikawa et al., 2012], or binding of miR-122 to the 3′UTR of SOCS3 mRNA and thereby a post-transcriptional SOCS3 inhibition [Gao et al., 2015]. SOCS3 inhibition by promoter methylation has been observed in several colorectal cancer cell lines, where the methyltransferase DNMT1 downregulates SOCS3 expression via hypermethylation in an IL-6 dependent manner [Li et al., 2012]. Blocking DNMT1 increases SOCS3 expression in unchallenged cells, rendering them susceptible to IL-6 induced SOCS3 upregulation. A SOCS3 downregulation by hypermethylation can also also be observed during the progression of lung
cancer in rats [Liu et al., 2010] and humans [He et al., 2003]. SOCS3 promoter analyses from various human HCC cell lines revealed aberrant methylation in three cell lines, and six out of 18 primary HCC samples [Niwa et al., 2005]. The responsible methylation site co-localizes with a conserved STAT binding site, approximately 500 bp upstream of the translation start site, providing a possibility for efficient ablation of STAT induced SOCS3 expression. Moreover, SOCS3 is a direct target of miR-483-5p, an intronic miRNA in the IGF2 locus, in murine Hepa1-6 hepatoma cells, substantiating SOCS3’s function both in metabolism as well as cancerogenesis [Ma et al., 2011]. Consistently, an upregulation of miR-802 under obese conditions indirectly increases SOCS3 expression by downregulating the transcription factor Hnf1b [Kornfeld et al., 2013]. Furthermore, diabetic patients exhibit a decrease in miR-185 plasma levels, negatively correlating with blood glucose levels and SOCS3 expression [Bao et al., 2015]. An inhibition of miR-185 in the pancreatic beta-cell line MIN6 results in a decreased glucose stimulated insulin secretion, reduced cell growth and viability and ultimately increased apoptosis. SOCS3 is a direct target of miR-185, as co-tranfection of a SOCS3-expressing plasmid and miR-185 restores the metabolic phenotype. miR-185 additionally has already been implicated in cancer progression of various human cancer entities, namely non small cell lung cancer [Takahashi et al., 2009], colorectal cancer [Liu et al., 2011], ovarian-, renal- and breast-cancer [Imam et al., 2010] and gastric cancer [Li et al., 2014], though no connection to SOCS3 is reported. A miR-203 knock-down in breast cancer tissues however enhances SOCS3 expression and increases apoptosis [Ru et al., 2011].

**SOCS3 function is indispensable for the developing organism**

SOCS3 functions at the heart of several highly important signaling pathways, and as such plays a crucial role in embryonic development. Consequently, a homozygous whole body knockout of SOCS3 is lethal between embryonic days 11 and 13 (E11-E13), due to placental deficiency [Roberts et al., 2001]. Conditional knockouts, using the Cre/loxP system, have been utilized to elucidate the contribution
of SOCS3 to individual cell types or signaling processes.

1.2.2 SOCS3 controls immune cell differentiation

Function of both the innate as well as adaptive immune system relies heavily on inflammatory signaling, whether immune cells are attracted by chemokine signaling to a site of infection or whether their maturation to elicit are more defined response is induced by interleukins or other cytokines. SOCS3 function, like that of other suppressors of cytokine signaling, is very important in many immune cell types. SOCS3 is constitutively expressed in CD4+ naïve T-cells and maintains their quiescent state \cite{Yu2003}. Stimulation of naïve T-cells with hen egg lysozyme decreases SOCS3 expression and concomitantly upregulates IL-2 production, which is in line with an observation that elevated SOCS3 action inhibits CD28-mediated IL-2 production and proliferation in T-cells via its SH2 domain \cite{Matsumoto2003}. Conflicting data demonstrate hyperproliferation of CD8+ T-cells from gp130\textsuperscript{Y757F}/gp130\textsuperscript{Y757F} mutant mice (where SOCS3 cannot bind to, and thereby inhibit signaling through, the gp130 receptor) in response to T-cell receptor (TCR) signaling, suggesting an impact of SOCS3 on IL-27 signaling rather than TCR- or CD28-mediated signaling to modulate T-cell proliferation \cite{Brender2007}. An inverse correlation between SOCS3 and IL-2 expression corroborates an implication for SOCS3 in T\textsubscript{H1} cell lineage commitment, where high levels of SOCS3 expression have been found in the T\textsubscript{H2} subtype, which does not express IL-2, and only very low levels in IL-2 secreting T\textsubscript{H1} cells \cite{Egwuagu2002}. SOCS3 function in T\textsubscript{H} cell lineage commitment is further substantiated by an enhanced T\textsubscript{H2} development, measured by enhanced IL-4 in combination with suppressed IFN-\(\gamma\) production, upon SOCS3 overexpression and consistently T\textsubscript{H2} suppression alongside SOCS3 haploinsufficiency \cite{Seki2003}. Enhanced SOCS3 action in T\textsubscript{H2} cells leads to a reduction of IL-12 induced STAT4 activation by binding to pY800 on IL-12R\(\beta\)2, subsequently inhibiting IFN-\(\gamma\) secretion and maintaining the T\textsubscript{H2} commitment \cite{Yamamoto2003}.
SOCS3 and $T_H17$ cell development

The classical concept of naïve CD4$^+$ T-cells developing either into $T_H1$ or $T_H2$ cells had to be modified with the recognition of IL-17 secreting CD4$^+$ T-cells as a separate subtype, consistently termed $T_H17$, which is negatively regulated by $T_H1$ and $T_H2$ cells [Harrington et al., 2005; Park et al., 2005]. Development of $T_H17$ cells is driven by dendritic cells secreting IL-23, a heterodimeric cytokine sharing subunit p40 with IL-12 and also signaling through the IL-12$\beta_1$ receptor [Aggarwal et al., 2003]. An investigation of the $T_H1/T_H2$ polarization in context of a SOCS3-deficiency via MMTV-cre (mammary tumor virus-cre) [Hennighausen et al., 1995] revealed no changes compared to the wild-type, but enhanced STAT3 phosphorylation upon IL-23 stimulation and a subsequent induction of IL-17 secretion [Chen et al., 2006]. Similar results were obtained when TGF-β stimulation of naïve CD4$^+$ T-cells increased $T_H17$ development by decreasing SOCS3 expression, thereby also prolonging pSTAT3 signaling [Qin et al., 2009]. An elevated secretion of TGF-β from SOCS3-deficient CD4$^+$ T-cells was already implicated in the development of $T_H3$ cells [Kinjyo, 2006], but later connected to an increased IL-17 production upon siRNA mediated SOCS3 down-regulation in CD4$^+$ T-cells [Moriwaki et al., 2011].

The inhibitory effect of SOCS3 on $T_H17$ T-cell development was further substantiated by LIF (leukemia inhibitory factor) treatment of CD4$^+$ T-cells and the subsequent SOCS3 and ERK upregulation, abrogating pSTAT3 signaling essential for $T_H17$ T-cell proliferation [Cao et al., 2011], as well as impaired IL-17 production upon SOCS3 overexpression in T lymphocytes [Romain et al., 2013].

$T_H$ cell proliferation is globally suppressed bei CD4$^+$CD25$^+$FoxP3$^+$ T$_{reg}$s, which do not show SOCS3 expression [Pillemer et al., 2007], despite their expression of and signaling through IL-6Ra [Doganci et al., 2005]. Stimulation of T$_{reg}$/T$_H$ co-cultures with IL-2 and IL-6 inhibits the T$_{reg}$-mediated T$_H$ cell suppression, due to dominant effects of the cytokines on T$_H$ cells. Further analyses demonstrated that, since SOCS3 mRNA is indeed expressed also in T$_{reg}$s, SOCS3 protein levels have to be kept low by post-translational modification. The inverse correlation of SOCS3
levels and IL-2 signaling observed in $T_H$ cells seems to also be reflected in $T_{reg}$s, where IL-2 signaling is crucial for their homeostatic maintenance [Fontenot et al., 2005]. Rapid SOCS3 upregulation in $T_{reg}$s upon infection could therefore mediate the release of $T_H$ cells from $T_{reg}$-mediated suppression. Furthermore, SOCS3 deletion in dendritic cells (DCs) selectively promotes FoxP3$^+$ $T_{reg}$s by increasing TGF-β1 production, suggesting SOCS3 as a key regulator of the DC-mediated balance between regulatory and effector T-cells [Matsumura et al., 2007].

SOCS3 promotes M1 macrophage polarization

Macrophages are part of the innate immune system, and act both as antigen-presenting cells (APCs) as well as mediators of the immune response of infiltrating leukocytes. Macrophages can be differentiated into „classically activated“ (M1) macrophages and „alternatively activated“ (M2) macrophages [reviewed by Mosser & Edwards, 2008]. M1 macrophages are considered anti-microbial and cytotoxic and are induced by a combination of IFN-γ and pro-inflammatory stimuli like LPS or TNF-α, whereas M2 macrophages function mainly in tissue-repair and are induced by IL-4 or IL-10. Polarization to either subtype renders the macrophage unresponsive to the inducers of the respective other subtype [Erwig et al., 1998]. In line with the differentiating functions of M1 vs. M2 macrophages, 80% of infiltrating macrophages in the acute stage of nephrotoxic nephritis express either SOCS1 or SOCS3, with the majority expressing SOCS3 [Liu et al., 2008]. Markedly, bone-marrow derived macrophages (BMDMs) stimulated with IFN-γ exclusively express SOCS3, whereas IL-4 stimulated BMDMs selectively upregulate SOCS1. Responsiveness to M2 inducing IL-4 signaling is restored upon siRNA mediated SOCS3 knock-down, demonstrating a function for SOCS3 in maintaining M1 polarized macrophages. A correlation between SOCS3 expression and M1 polarization is substantiated by a strong co-expression of SOCS3 and the M1 marker iNOS (nitric oxide synthase) in infiltrating, glomerular macrophages in nephritis [Arnold et al., 2014]. siRNA mediated SOCS3 depletion results in an upregulation of M2 markers
like arginase-1 or mannose receptor. A potential mechanism for unresponsiveness of M1 macrophages to M2 inducing stimuli in context of SOCS3 activity is provided by observations from peritoneal macrophages obtained from db/db mice, where IL-4 induced, IRS-2 (insulin receptor substrate)/PI3K (phosphoinositide 3-kinase)-association-dependent upregulation of IL-1Rα is attenuated by a chronic SOCS3 overexpression [O’Connor et al., 2007]. Consistently, macrophages from IL-4Rα-/- mice showed an upregulation in SOCS3 protein levels [Whyte et al., 2011]. Conflicting data show an enhanced M1 macrophage polarization upon myeloid-specific SOCS3 deletion, although an upregulation of SOCS3 in macrophages upon M1 inducing cytokine stimulation is confirmed [Qin et al., 2012]. Furthermore, a myeloid-specific deletion of SOCS3 promotes both T_{H}1 and T_{H}17 response of CD4+ T-cells. Interestingly, responsiveness of SOCS3 deficient macrophages to IL-4 stimulation is not assessed.

T_{H}1/2 cytokines IFN-γ and IL-4 are also released in large amounts by stimulated NK-T cells, which are an abundant T-cell subpopulation in the liver. A lymphocytic overexpression of SOCS3 using the lck (lymphocyte specific protein tyrosine kinase)-promoter in T and NK-T cells (Lck-SOCS3 tg) results in decreased serum IFN-γ and IL-4 levels, without affecting lymphocyte numbers oder cytotoxic activity, when Lck-SOCS3 tg mice are subjected to a Concanavalin-A (ConA) induced model of hepatitis [Nakaya et al., 2009]. Lck-SOCS3 tg mice are protected from ConA induced hepatitis, and exhibit decreased phosphorylation of STAT1 and STAT3 specifically in NK-T cells, and not in T-cells. This leads to suppression of cytokine release from NK-T cells, ultimately suggesting a protective effect against liver injury for SOCS3 expression in NK-T cells, as opposed to SOCS3 action in hepatocytes, where a deletion of SOCS3 protects against ConA induced hepatitis [Ogata et al., 2006b].

1.2.3 SOCS3 regulates energy homeostasis

The regulation of energy homeostasis is a very complex machinery, encompass-
ing the central control by the arcuate nucleus in the hypothalamus, as well as peripheral control in tissues like liver, skeletal muscle or adipose tissue. Abundant energy in form of elevated blood glucose levels triggers the release of insulin from the pancreas, which signals both on neurons in the arcuate nucleus to modify food intake as well as on peripheral tissues like the liver, to suppress gluconeogenesis, or skeletal muscle to take up glucose. With food intake constantly exceeding energy consumption, ever increasing amounts of adipocytes will be stored in the adipose tissue, which correlates with their proportional secretion of leptin, the „satiety hormone“. The intricate network of energy homeostasis is under extensive investigation for many decades now and reviewed constantly and manifold [e.g. Karatsoreos et al., 2013; Friedman, 2014].

Both insulin and leptin upregulate SOCS3 expression, and both their intracellular signaling cascades are in turn regulated by SOCS3. A deletion of SOCS3 in neuronal precursor cells with nestin-cre or synapsin-cre results in increased and prolonged tyrosinephosphorylation of STAT3 upon leptin administration [Mori et al., 2004]. This enhanced leptin sensitivity leads to increased expression of pro-opiomelanocortin (POMC) in neurons, which secrete the anorexigenic neuropeptide α-MSH. Consequently, leptin administration results in reduced food intake and a greater weight loss in mice with neuronal SOCS3 deficiency. Furthermore, weight gain upon HFD feeding is decreased and insulin sensitivity maintained in the absence of SOCS3 in neurons. A more specific deletion of SOCS3 in POMC neurons of the arcuate nucleus results in a very similar phenotype, namely enhanced leptin sensitivity and glucose tolerance upon NCD and reduced weight gain, due to increased energy expenditure, and improved insulin sensitivity on HFD [Kievit et al., 2006]. Another brain region involved in feeding behavior is the ventromedial hypothalamus (VMH), whose neurons abundantly express steroidogenic factor 1 (SF-1). Deletion of SOCS3 with a SF-1-cre consistently leads to enhanced leptin sensitivity, reduced food intake and improved insulin sensitivity [Zhang et al., 2008].

As for the peripheral impact on energy homeostasis, obesity has been increas-
ingly recognized as a state of chronic low grade inflammation [Hotamisligil et al., 1993; Xu et al., 2003]. Increased circulating cytokine levels under obese conditions, as assessed in mice lacking the leptin receptor (db/db mice), subsequently upregulate SOCS3 in hepatocytes [Ueki et al., 2004a]. Increased expression of SOCS3 in the liver inhibits insulin signaling by blocking the IRS1 binding site pY960 on the insulin receptor, resulting in insulin resistance [Emanuelli et al., 2001]. Consequently, a hepatocyte specific SOCS3 knock-out increases insulin stimulated IRS1 phosphorylation [Senn et al., 2003] and thereby protects from IL-6 induced insulin resistance [Torisu et al., 2007; Sachithanandan et al., 2010], whereas an overexpression of SOCS3 leads to insulin resistance by reducing IRS1 and IRS2 phosphorylation [Ueki et al., 2004b].

The protective effect on energy homeostasis of attenuated SOCS3 function in hypothalamus and liver is, to a lower extent, also present in other peripheral tissues. A knockout of SOCS3 in the white adipose tissue (WAT) shows only a mild protection of obesity induced insulin resistance in female mice [Palanivel et al., 2012], while a SOCS3 deletion in skeletal muscle results in an increased peripheral glucose disposal and partially protection from insulin resistance [Jorgensen et al., 2012]. Furthermore, a whole body SOCS3 haploinsufficiency (SOCS3+/−) conveys enhanced leptin sensitivity and protection from diet-induced obesity [Howard et al., 2004].

1.2.4 SOCS3 is heavily involved in cancerogenesis

The interplay between metabolism, inflammation and cancer puts SOCS3 in a key position to influence tumorigenesis. As a hepatocyte specific SOCS3 deletion in the ConA-induced hepatitis model, prolonging intracellular signaling after cytokine stimulation, protects from liver injury by upregulating Bcl-XL and thereby decreasing apoptosis, loss of SOCS3 in hepatocytes and the resulting escape from apoptosis exacerbates DEN-induced HCC formation [Ogata et al., 2006b]. A similar aggravation of DEN-induced HCC formation can be observed upon SOCS3 hap-
loinsufficiency. Interestingly, SOCS3 depletion in both liver parenchymal as well as non-parenchymal cells together exacerbates not only HCC formation, but also ConA-induced hepatitis via TGF-β1 upregulation [Ogata et al., 2006a]. Consistently, a hepatocyte specific gp130 knock-out ameliorates acute inflammation upon high-dose DEN-injection, demonstrated by decreased serum ALT/AST (alanine and aspartate transaminases) levels as well as less IL-6 and oncostatin M (OSM) in liver protein lysates [Hatting et al., 2015]. HCC initiation 24 weeks after regular-dose DEN-injection is unchanged compared to wt, but differences are visible in HCC progression, 40 weeks after DEN-injection. Tumor tissue shows less nodules, a tendency for less tumor associated inflammation, less DNA damage and decreased TGF-β expression.

A similar, tumor-suppressive function for SOCS3 has been assigned in colorectal cancer (CRC) formation. An intestinal epithelial cell (IEC)-specific SOCS3 deletion (SOCS3IEC-KO) results in a strong hyperproliferation and hyperplasia after treatment with colitis-inducing DSS [Rigby et al., 2007]. Consistently, SOCS3 overexpression in intestinal epithelial cell lines inhibits proliferation. Additional AOM injection into DSS-treated SOCS3IEC-KO mice results in increased tumor formation, initiated by elevated levels of nuclear pSTAT3 and NF-κB, ultimately increasing IEC proliferation and tumorigenesis. Risk of CRC formation, among other cancers, has additionally been found to be elevated in obesity [reviewed in Renehan et al., 2008]. Interestingly, genetically obese, leptin-deficient (ob/ob) mice subjected to the AOM/DSS model develop more tumors, comparable to HFD-fed wild-type animals, than lean animals, but have a decreased tumor size at later stages despite overt obesity [Endo et al., 2011]. Consistently, LepR-deficient (db/db) mice also exhibit a decreased tumor frequency and size compared to HFD-fed animals, despite being obese. The protective effect of loss of leptin signaling is mediated by lack of pSTAT3 activation and subsequently decreased activation of proliferation in concert with increased apoptosis. Similar observations can be made for gastrointestinal epithelial cell-specific SOCS3 deletion, initiating hyperproliferation via continuos
leptin signaling and subsequent pSTAT3 activity [Inagaki-Ohara et al. 2014]. Furthermore, leptin signaling has been found to impact on CD133⁺Nanog⁺ Tumor initiating stem cells (TISCs), which strongly express the LepR [Feldman et al. 2012], providing a mechanism how increased serum leptin levels under obese conditions can promote tumorigenesis.

1.3 Site specific recombination & conditional gene targeting

Biological and medical research nowadays heavily relies on the use of genetically modified organisms to study role and function of genes, both protein-coding and non-coding. A series of giant leaps in laboratories around the world in the last 40 years advanced the field from studying individual organisms with random, naturally occurring mutations to custom made, gene-targeted mice harboring artificially introduced mutations with nucleotide precision [establishment of gene targeting extensively reviewed by Mario Capecchi, 2005]. The specific disruption of any target gene, now termed conventional gene targeting, is a vastly powerful tool to examine gene function, taking full advantage of the sequenced mouse genome [Mouse Genome Sequencing Consortium et al. 2002]. But even this technique, unthinkable mere 30 years ago, has certain limitations when it comes to a very fine tuned analysis of gene functions or the investigation of essential genes. Utilizing site specific recombinase (SSR) systems in gene targeting, termed conditional gene targeting, can alleviate restrictions imposed by conventional gene targeting [Kühn et al. 1995; Rajewsky et al. 1996].

1.3.1 Mechanism of site specific recombination

With the use of conditional gene targeting and site specific recombination, gene disruption is elevated from a basic, whole body deletion to a more sophisticated control of the genetic modification, including excision, insertion or inversion of DNA fragments with full spatial and temporal control. Site specific recombinases can be found in several organisms where they have varying functions. SSR systems
Site specific recombination requires two recombinase target sites, indicated by black arrows, within the DNA and can either result in excision/insertion or inversion, determined by the relative orientation of the target sites. (a) Two target sites in tandem orientation will result in the excision of the flanked DNA regions, b and c, as a circular DNA fragment with one target site and the other target site within the remaining DNA. Insertion of the circular DNA fragment is possible but unfavored. (b) Two target sites facing each other will result in an inversion of the flanked DNA regions, f and g, and an otherwise unchanged DNA structure, enabling further inversions of the flanked DNA regions. [modified from Branda & Dymecki, 2004]

most commonly used in transgenic mice are members of the λ integrase superfamily of SSRs, like Cre/loxP from bacteriophage P1 [Sternberg & Hamilton, 1981] or Flp/frt from Saccharomyces cerevisiae [Andrews et al., 1985]. Recombination by both Cre and Flp follow a common mechanism (Fig. 1.5): Site specific recombinase tetramers recognize 34 bp DNA regions, composed of two 13 bp palindromic sequences, called inverted repeats, flanking an 8 bp non-palindromic core sequence, called spacer, determining the overall orientation of the recombinase target site. Recombination requires two such target sites, where the recombinase introduces strand cleavage, exchange and ligation. The target site orientation determines the exact recombination event, either facilitating strand excision/insertion in case of two target sites facing the same direction, or strand inversion in case of two target sites facing each other. An inversion reaction can occur multiple times, since the DNA structure is not changed after the recombination event.

Generally, a combination of two transgenic mouse lines is used to utilize site
specific recombination and conditional gene targeting in mice. One mouse will
express the respective recombinase, using a specific promoter to determine the site
of recombination. The promoter will either convey a spatial control of recombi-
nase expression, differentiating between so called deleter-strains, expressing the
recombinase under a ubiquitous promoter, vs. expressing the recombinase under
a tissue-specific promoter. Recombinase expression can also be under temporal
control using an inducible promoter, to allow targeting of genes essential for de-
velopment. By using post-translational methods of protein induction, spatial and
temporal control of recombinase activity can even be combined. Taken together,
expression of the recombinase usually determines the „condition“ of conditional
gene targeting.

A second mouse harbors recombinase target sites within its genome, whose re-
combination can have various effects. A conditional knock-out allele would have
several exons flanked by recombinase target site to induce strand-excision in the
presence of the recombinase, ideally introducing a frameshift in the open reading
frame to completely abolish gene transcription. A conditional knock-in allele on
the other hand would have a transcriptional STOP-cassette flanked by recombinase
target sites introduced between the promoter and the first exon, enabling tran-
scription of the downstream target gene only in the presence of the recombinase.
Recombinase target sites facing each other are usually used to switch the expres-
sion of one particular gene to another, this way either exchanging gene of interest
expression with expression of a reporter gene, or a mutated version of the same
gene.

1.3.2 Dre/rox is a novel recombinase system distinct from Cre/loxP

The Cre/loxP system is the undisputed incumbent of the site specific recombi-
nases, with the vast majority of conditional mouse lines employing Cre driver lines
and loxP-based target alleles. Cre excels at both efficiency as well as versatility,
when compared to other tyrosine recombinases like Flp or the larger serine recom-
binases such as ΦC31. Flp matches Cre in versatility, being able to mediate excision/insertion as well as inversion, but, as a protein from Saccharomyces cerevisiae, is significantly less efficient in mammalian cells, due to a decreased thermostability [Buchholz et al., 1996]. A mutated form Flp-e with increased thermostability shows improved efficiency [Buchholz et al., 1998], but still does not match Cre. Large serine recombinases like ΦC31 not only lack some of Cre’s high efficiency, but also are less versatile, in that they only mediate a directed, irreversible integration [Belterki et al., 2003], rendering them well suited for cassette exchange strategies, but not to cover the diverse applications of Cre. Recently de novo synthesized, codon-optimized versions of Flp and ΦC31, termed Flp-o and ΦC31-o, further raise their efficiency to be „similar to Cre“ but, especially for Flp-o, still cannot quite match it [Raymond & Soriano, 2007].

Sequencing of four P1-related phages, which are maintained as an extrachromosomal plasmid like P1 and should therefore also have a recombinase system to resolve phage DNA dimers after replication [Austin et al., 1981], to identify Cre homologs revealed a closely related site specific recombinase system in the phage D6, termed Dre/rox [Sauer & McDermott, 2004]. Dre recombinase shares 39% similarity with Cre recombinase, and requires the unique rox target site for recombination. The 32 bp rox site consists of two 14 bp inverted repeats, separated by a directional 4 bp spacer, as compared to the 34 bp loxP site, with about 55% sequence homology to loxP. The Dre/rox system was subsequently adopted and analyzed further in bacteria, in vitro and in vivo [Anastassiadis et al., 2009]. Complete recombination between rox sites in bacteria is only achieved in the presence of Dre recombinase, neither in the absence of Dre, nor in presence of Cre recombinase. Likewise, Dre recombinase does not mediate recombination between loxP sites. Similar results were obtained with eukaryotic expression vectors and cell lines in vitro, successful recombination happens exclusively for the Cre/loxP combination or Dre/rox, respectively. The combined efficiency and specificity of Dre/rox was also observed in vivo (Fig. 1.6). Lastly, an inducible Dre system was constructed utilizing the mod-
Figure 1.6: **Dre specificity in vivo**
CAGGs-Dre and CAGGs-Cre mice, expressing the respective recombinase under the ubiquitous CAGGs promoter, were either crossed to ZEG mice, expressing lacZ before and GFP after successful excision of a loxP-flanked STOP-cassette, or Rosa-rox-lacZ mice, expressing β-galactosidase after successful excision of a rox-flanked STOP-cassette. (a) LacZ staining and GFP epifluorescence indicate that Dre recombinase does not recombine loxP sites in mice. (b) Likewise, Cre does not recombine rox sites in mice. [modified from Anastassiadis *et al.*, 2009]

1.3.3 **Generation of transgenic mice by BAC recombineering**

The utility of conditional mouse models and the usage of site specific recombinases critically depends on the specificity of the recombinase driver line. This becomes especially evident if the recombinase is supposed to be expressed under the control of a tissue-specific promoter, where it is important that every regulatory promoter element is functional. Red/ET cloning, also called recombineer-
ing, is a rapid procedure to create a tissue-specific, transgenic mouse line [Zhang et al., 1998]. Bacterial artificial chromosomes (BACs) are available ready-to-buy in a host *E. coli* strain from the BACPAC Resources Center. By introducing a plasmid containing the recombination proteins Redα and Redβ coding sequences from bacteriophage *λ* into the *recA* host bacteria, the BAC carrying bacteria are recombination competent if expression from the plasmid is induced by arabinose addition [Muyrers et al., 1999]. The gene of interest, in this case the recombinase, is amplified by PCR with 50 bp homology arms corresponding to the translation-start of the respective driver gene on the BAC. Successful recombination will result in a modified BAC, expressing the recombinase under the full, endogenous promoter of at least 5 kbp upstream of the transcription-start. Pronucleus injection of the modified BAC gives rise to a founder line with full penetrance upon successful BAC integration into the genome.
1.4 Objectives

In recent decades, Inflammatory signaling has become increasingly recognized as a key system both under cancerous as well as obese conditions. Our immune system is struggling to keep a very delicate balance between initiating cell death and promoting tissue remodeling by inducing cell proliferation. In this regard, members of the innate as well as the adaptive immune system secrete a large variety of inflammatory mediators, cytokines and chemokines, into target tissues and the blood stream, signaling on parenchymal and non-parenchymal cells alike. Only the complex interaction of the various inflammatory mediators present, the source of the respective cytokine or chemokine and the exact target cell, determines whether innate and adaptive immune response act in concert or with diametral consequences.

In order to analyze the role of inflammatory signaling of the various immune cell subpopulations in cancer initiation, promotion and progression, as well as to monitor cancer cell fate, this study aims to develop a universal reporter tool to visualize inflammatory signaling both in vivo and in vitro. Although different cancer entities can vary greatly in onset, severity and mortality, there are certain hallmarks on the cellular level which can be exploited. Suppressor of cytokine signaling 3 (SOCS3) is a negative feedback regulator of, among others, the JAK/STAT signaling pathway, and its expression is upregulated by a plethora of inflammatory mediators. Inflammatory signaling can therefore be visualized by utilizing the endogenous SOCS3 promoter to drive expression of reporter proteins.

Aim of this thesis is the generation of the universal reporter mouse line SOCS3-U and to use it in mouse models of chemically induced cancerogenesis. Utilizing SOCS3-U in combination with different Cre-driver lines to activate only specific immune cell subpopulations, this study should give a more detailed insight into the contribution of the respective cell types, its inflammatory mediators and their impact on cancer initiation, promotion and progression.
2 Materials and Methods

Standard methods of molecular biology were performed according to established protocols [Green & Sambrook, 2012] if not stated otherwise.

2.1 Genetic Engineering

Cloning of polynucleotides was done using PCR, restriction digest and DNA ligation (T4 DNA ligase, NEB, Frankfurt, Germany). Table 2.2 lists all primers utilized for cloning and table 2.3 lists all restriction endonucleases. Amplicons generated by PCR were cloned into the pGEM-T-Easy vector system (Promega, Madison, WI, USA) and sequenced if not stated otherwise. Sequencing was done by GATC Biotech (Konstanz, Germany) either utilizing T7 and SP6 primers provided by the company for sequencing of pGEM inserts, or using the primers listed in table 2.4.

All cloning procedures were performed with XL-10 Gold Ultracompetent cells (Agilent, Santa Clara, USA). Bacteria were cultivated in LB-medium or on LB-agar at 37°C if not stated otherwise. Table 2.1 contains a list of contents for bacterial cultures.

<table>
<thead>
<tr>
<th>Table 2.1: Bacterial Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Content</strong></td>
</tr>
<tr>
<td>LB</td>
</tr>
<tr>
<td>LB-agar powder</td>
</tr>
<tr>
<td>Ampicillin 50 mg/ml H2O</td>
</tr>
<tr>
<td>Tetracycline 5 mg/ml 70% EtOH</td>
</tr>
<tr>
<td>Chloramphenicol 20 mg/ml 70% EtOH</td>
</tr>
<tr>
<td>Kanamycin 50 mg/ml H2O</td>
</tr>
<tr>
<td>X-Gal 40 mg/ml DMF</td>
</tr>
<tr>
<td>IPTG 0.1 M in H2O</td>
</tr>
<tr>
<td><strong>Stock</strong></td>
</tr>
<tr>
<td>powder</td>
</tr>
<tr>
<td>powder</td>
</tr>
<tr>
<td>50 mg/ml H2O</td>
</tr>
<tr>
<td>5 mg/ml 70% EtOH</td>
</tr>
<tr>
<td>20 mg/ml 70% EtOH</td>
</tr>
<tr>
<td>50 mg/ml H2O</td>
</tr>
<tr>
<td>40 mg/ml DMF</td>
</tr>
<tr>
<td>0.1 M in H2O</td>
</tr>
<tr>
<td><strong>Working concentration</strong></td>
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<tr>
<td>25 g/l</td>
</tr>
<tr>
<td>35 g/l</td>
</tr>
<tr>
<td>50 μg/ml</td>
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<tr>
<td>50 μg/ml</td>
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<td>20 μg/ml</td>
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<tr>
<td>50 μg/ml</td>
</tr>
<tr>
<td>40 μg/ml</td>
</tr>
<tr>
<td>0.2 mM</td>
</tr>
</tbody>
</table>
2.1.1 Cloning of targeting constructs

Cloning PCRs were done with the High Fidelity PCR Master (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Standard reaction conditions generally were: 50 µl reaction mix, containing 50 pmol of each primer and at least 50 ng DNA template. The thermocycler was programmed according to the specific PCR with an annealing temperature fitting the respective primers and an elongation time of 1 min/kb amplicon.

<table>
<thead>
<tr>
<th>Description</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS3-U</td>
<td>5MluRL</td>
<td>AAAACGCCTTAAAATATGACAGATTATT TAAAGTTCGCGGCAATGACGCGAGG</td>
</tr>
<tr>
<td></td>
<td>3MluRL</td>
<td>ACCGCGTTAATTTAAATATGCGAAATTATTTAA AGTTATGGCTCATTGAAAGAAGCTG</td>
</tr>
<tr>
<td></td>
<td>5RsrLA</td>
<td>AAAACGGTCCGAAGCTTAGACTGGGAGTCTAAT TTCTTCAGAGCATCGAGGAGG</td>
</tr>
<tr>
<td></td>
<td>3PacLA</td>
<td>TTTTTAATTAAGATAATCCACACACAGAGTCT TCTT</td>
</tr>
<tr>
<td></td>
<td>5RVRA</td>
<td>AAAAGATATCGGCGCCGCGGTTAAAACATTTAA AATGGGAAAAAGGTGAGGAGG</td>
</tr>
<tr>
<td></td>
<td>3RVRA</td>
<td>TTTACCAGTATCCAGGGCTGAGGAGG</td>
</tr>
<tr>
<td></td>
<td>5AgeRA</td>
<td>AAAACGGTATCGGCGCCGCGGTAAAAA</td>
</tr>
<tr>
<td></td>
<td>3AgeRA</td>
<td>TTTACCAGTATCCAGGGCTGAGGAGG</td>
</tr>
<tr>
<td></td>
<td>5SwaLuc</td>
<td>AAAATTTAAATGAAGTTCCTATTGCAAGTTC TATTCTTAGAAAGATAGGAACCTTCCTTTGC TCTGACAGGCCCACCATGGAAAGACGCACCAA</td>
</tr>
<tr>
<td></td>
<td>3AscLuc</td>
<td>AAAAGCGCGCCCTTAAACTTACAATTTGAGGACTT TCCGCG</td>
</tr>
<tr>
<td>Alb-Dre</td>
<td>5AlbDre</td>
<td>TGTGGTGCTGGTTTTTCTCTCACTTTCCACAG ACAAGAGTGAGATCGCCACCATGGGTAAGAA GAAGA</td>
</tr>
<tr>
<td></td>
<td>3AlbDre</td>
<td>ATACCTACAGGGCTTTGGAATGTTGTTTCTCC AAAATCATATACCGGATGGAAGATTATAATTTC TACGCG</td>
</tr>
<tr>
<td></td>
<td>5Alb</td>
<td>GTCTCCGGCTCTGTTTTTCCAGG</td>
</tr>
<tr>
<td></td>
<td>3Alb</td>
<td>TGTTTCCTAAAATCAATTATACCG</td>
</tr>
<tr>
<td></td>
<td>3Dre</td>
<td>TACTCCCTAGCCCATCTCAGGAGAGAT</td>
</tr>
</tbody>
</table>
Restriction digest was either performed in a 50 μl reaction mix for cloning or 10 μl reaction mix for analysis. Analytic restriction digests contained around 500 ng DNA, restriction digests for cloning around 5 μg DNA. Linearized DNA vectors were dephosphorylated using Antarctic phosphatase (NEB, Frankfurt, Germany). Separation of DNA fragments was done by agarose gel electrophoresis. DNA fragments were extracted with the QIAEX II gel extraction kit (Qiagen, Hilden, Germany).

Table 2.3: Restriction Endonucleases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Supplier</th>
<th>Recognition Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgeI-HF</td>
<td>NEB</td>
<td>A</td>
</tr>
<tr>
<td>AscI</td>
<td>NEB</td>
<td>GG</td>
</tr>
<tr>
<td>AsiSI</td>
<td>Fermentas</td>
<td>GCGAT</td>
</tr>
<tr>
<td>AvrII</td>
<td>NEB</td>
<td>C</td>
</tr>
<tr>
<td>EcoRV-HF</td>
<td>NEB</td>
<td>GAT</td>
</tr>
<tr>
<td>PacI</td>
<td>NEB</td>
<td>TTAAT</td>
</tr>
<tr>
<td>PvuI-HF</td>
<td>NEB</td>
<td>CGAT</td>
</tr>
<tr>
<td>SwaI</td>
<td>NEB</td>
<td>ATTT</td>
</tr>
<tr>
<td>PI-SceI</td>
<td>NEB</td>
<td>homing endonuclease</td>
</tr>
</tbody>
</table>

For sequencing analysis, 500 ng DNA [20 ng/μl] were sent to GATC Biotech. Custom primer were sent at 10 μM concentration.

Table 2.4: Sequencing Primer

<table>
<thead>
<tr>
<th>Description</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS3-U</td>
<td>5SOCS1</td>
<td>GTGCGCAAGCTGCAGGAGAG</td>
</tr>
<tr>
<td></td>
<td>5SOCS2</td>
<td>TGTGTACTCAAGCTGGTGCA</td>
</tr>
<tr>
<td></td>
<td>5SOCS3</td>
<td>GTAGCTCCAGTGAGCCAGG</td>
</tr>
<tr>
<td></td>
<td>5LA1</td>
<td>CTATCACAGTGTCACCTG</td>
</tr>
<tr>
<td></td>
<td>5LA2</td>
<td>ATCTCCATCTGGAACATC</td>
</tr>
<tr>
<td></td>
<td>5LA3</td>
<td>CTTCAATCCTGCTCTCTC</td>
</tr>
<tr>
<td></td>
<td>5LA4</td>
<td>GGTGATAAGGTAGTTAGT</td>
</tr>
<tr>
<td></td>
<td>5LA5</td>
<td>CTGCCAGAAAACCAGCCTT</td>
</tr>
<tr>
<td></td>
<td>5LA6</td>
<td>CAGCTCTCGTGAGGTCC</td>
</tr>
<tr>
<td></td>
<td>5LA7</td>
<td>TCGGCCACTGAGGACACCGGA</td>
</tr>
<tr>
<td></td>
<td>5RA0</td>
<td>TAAACATTTTAATGGACAA</td>
</tr>
<tr>
<td></td>
<td>5RA1</td>
<td>GAACTTGTTTGCGCTTTGAT</td>
</tr>
</tbody>
</table>

Continued on next page
Table 2.4 – continued from previous page

<table>
<thead>
<tr>
<th>Description</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>5RA2</td>
<td>GGCTAGGAGACTCGCCTTAA</td>
<td></td>
</tr>
<tr>
<td>5Luc1</td>
<td>CAGTAAAGCTATGTCCTCCAGA</td>
<td></td>
</tr>
<tr>
<td>5Luc2</td>
<td>GTTGGTACTAGCAACGCCT</td>
<td></td>
</tr>
<tr>
<td>5Luc3</td>
<td>TAGAATCCATGATAATAATT</td>
<td></td>
</tr>
<tr>
<td>5Luc4</td>
<td>CGTATCTCTTCATAGCCTTA</td>
<td></td>
</tr>
<tr>
<td>3Luc1</td>
<td>ATATGTGCATCTGTAAAGC</td>
<td></td>
</tr>
<tr>
<td>3Luc2</td>
<td>CCGGTATGTAAACAATCCGGA</td>
<td></td>
</tr>
<tr>
<td>Alb-Dre</td>
<td>5Alb</td>
<td>GTCTCCGGCCTGCTTTTTCCAGG</td>
</tr>
<tr>
<td></td>
<td>3AmplifyFlp</td>
<td>AGCCAGAAAGTCAGATGCTCA</td>
</tr>
<tr>
<td>DreSeqJ</td>
<td>TGCTGTCTAGATCTGAGAGCT</td>
<td></td>
</tr>
<tr>
<td>DreSeq0j</td>
<td>CCTGTTATTTTTAAAATAAGT</td>
<td></td>
</tr>
<tr>
<td>DreSeq1j</td>
<td>TCTCATAGGGCTGCTGCT</td>
<td></td>
</tr>
<tr>
<td>DreSeq2j</td>
<td>TAGCTTAGTGTCAGTGAAGAG</td>
<td></td>
</tr>
<tr>
<td>DreSeq3</td>
<td>TCCACTCCTGGGCTAGATGG</td>
<td></td>
</tr>
<tr>
<td>DreSeq4</td>
<td>GTGGGAGACCTGGACCAGAC</td>
<td></td>
</tr>
<tr>
<td>DreSeq5</td>
<td>AAAGCCCTGAGACACTGAAG</td>
<td></td>
</tr>
<tr>
<td>3DreSeq</td>
<td>TCTCCGGATTTCTCCTCATG</td>
<td></td>
</tr>
<tr>
<td>Drerevneu</td>
<td>GCGGTGGTCTCTCTAGAC</td>
<td></td>
</tr>
</tbody>
</table>

SOCS3-U targeting construct

SOCS3 CDS was amplified using the primers 5MluRL and 3MluRL from genomic DNA and ligated into the pGEM-T-Easy Vector System. The amplicon was cut from the pGEM backbone with MluI and ligated with the common Stop-eGFP-ROSA-CAGs [SERCA, Klisch, 2006] plasmid, cut with AciI. The resulting intermediate was cut with NheI and EcoRV and the 5.7 kb fragment was ligated with the GK12TK plasmid [Wunderlich et al., 2010], cut with AvrII and PmeI. The SOCS3 LAH was amplified using the primers 5RsrLA and 3PacLA from genomic DNA. The amplicon was cut from the pGEM backbone with PvuI and PacI and inserted into the GK12TK-SOCS3 intermediate, cut with PacI. The SOCS3 RAH was amplified using the primers 5RVRA and 3RVRA from genomic DNA, which served as template for a second amplification using the primers 5AgeRA and 3AgeRA. Firefly luciferase CDS was amplified using the primers 5SwaLuc and 3AscLuc from the pTE-Luc plasmid [Jordan et al., 2011]. The amplicon was cut from the pGEM back-
bone with Ascl and SwaI and inserted into the pGEM-RA intermediate, cut with Ascl and SwaI as well. The RA-Luc intermediate was cut from the pGEM backbone with AgeI and inserted into the GK12TK-SOCS3-LA intermediate, cut with AgeI as well.

**Alb-Dre recombinant BAC**

Construction of the Alb-Dre recombinant BAC was performed via Red-E/T recombination [Muyrers et al., 1999]. Dre CDS and a neomycin resistance cassette were amplified using the primers 5AlbDre and 3AlbDre from the pTE-Dre-neo/kana [Tim Klöckener, University of Cologne] plasmid. The resultant DNA fragment contains 50 bp homology arms with the endogenous Alb locus.

1.4 ml LB medium containing chloramphenicol were inoculated with 30 µl overnight culture of Alb-BAC carrying bacteria and cultivated for 3 h at 37°C. Cells were centrifuged for 30 sec at 11,000 xg, 4°C. Cells were washed with 1 ml chilled water and centrifuged again. The cell pellet was resuspended in 20-30 µl chilled water. Resuspended cells were mixed with 3 µl pSC101-BAD-gbaA [100-200 ng/µl]. Cells were transformed via electroporation (1350 V, 10 µF, 600 Ω). Electroporated cells were resuspended in 1 ml LB medium and incubated for 70 min at 30°C. Cells were plated on LB agar plates containing tetracycline and chloramphenicol and incubated over night at 30°C. Colonies were picked and incubated in LB medium containing tetracycline and chloramphenicol over night at 30°C. 1.4 ml LB medium containing tetracycline and chloramphenicol were inoculated with 30 µl overnight culture and incubated at 30°C until an OD$_{600}$ of 0.15 was reached. 20 µl L-Arabinose were added and the cells incubated for 60 min at 30°C. Cells were then incubated at 37°C until an OD$_{600}$ of 0.4 was reached. Cells were centrifuged for 30 sec at 11,000 xg, 4°C. Cells were washed with 1 ml chilled water and centrifuged again. The cell pellet was resuspended in 20-30 µl chilled water. Resuspended cells were mixed with 3 µl linearized, recombinant DNA fragment [100-200 ng/µl]. Cells were transformed via electroporation (1350 V, 10 µF, 600 Ω). Electroporated cells were
resuspended in 1 ml LB medium containing 20 µl L-Arabinose and incubated for 70 min at 37°C. Cells were plated on LB agar plates containing kanamycin and incubated over night at 37°C. Colonies were picked and checked for correct insertion of the recombinant fragment. Recombinant BACs were linearized with PI-SceI and purified with the NucleoBond® Xtra BAC kit (Macherey-Nagel, Düren, Germany) for transfection.

2.1.2 Cell culture

All cells utilized were maintained in an incubator with constant conditions (95% humidity, 37°C, and 10% CO₂ saturation), while handling and passaging of cells was performed under a sterile hood (Hera Safe KS 12, Heraeus Instruments). Bruce4 embryonic stem cells [ES-cells, Köntgen & Stewart, 1993] were used for all transfection and kept on a confluent layer of mouse embryonic fibroblasts (MEFs). MEFs were passaged three times and treated with mitomycin C (MMC, Sigma-Aldrich) to serve as feeder cells for ES cells to maintain their pluripotency. Growth medium for ES cells was changed every day and every 2-3 days in case of MEFs. Contents of the various growth media are listed in table 2.5.

Passaging of cells

MEFs were passaged upon confluence, ES-cells at 85% confluency or at least every three days to maintain pluripotency. Cells were washed twice with PBS and incubated with trypsin-solution (0.05% trypsin, 0.02% EDTA in PBS) at 37°C for 5 min. Trypsin digest was stopped with 1:1 FCS-containing medium. Cells were centrifuged (270 xg, 5 min, 4°C) and seeded on fresh plates. MMC-treated MEFs were seeded on gelatin-coated (0.2% gelatin in PBS, ≥ 5 min at 37°C) plates to enhance adherence. Cells were counted in a C-Chip Neubauer improved counting chamber (Peqlab, Erlangen, Germany) if indicated.
Freezing and thawing of cells

For cell freezing, cells were trypsinized and centrifuged as described before. The cell pellet was resuspended in FCS containing 10% DMSO and stored at -80°C for short term storage or liquid nitrogen for long term storage. Frozen cells were thawed 1:40 in the appropriate culture medium, centrifuged and seeded in fresh medium on the appropriate dish.

Transfection

Medium of the ES-cells was changed 2-3 hours prior to transfection. 40 µg targeting construct were linearized with AsiSI, purified with isopropanol precipitation and resuspended in 400 µl PBS under sterile conditions. 10⁷ ES-cells were trypsinized and also resuspended in 400 µl PBS. ES-cells and linearized plasmid were mixed in an electroporation cuvette (0.4 cm) for transfection (230 V, 500 µF, ∞ Ω; GenePulser Xcell™, Bio-Rad, München, Germany). Cells were incubated for 5 min at RT and subsequently resuspended in ES-medium and seeded on four 10 cm dishes with feeder cells. Selection with G418 and counterselection with ganciclovir for correct integration of the targeting construct started two days post transfection and was carried out for seven days. Picking of colonies was performed nine days post transfection.

Picking of colonies

ES-cells were washed twice with PBS prior to picking colonies. Cells were picked in 40 µl PBS and transferred to a 96-well plate (round bottom) containing 25 µl trypsin solution. Picking was stopped once the 96-well plate was full or at least 30 minutes after picking the first colony. Cells were incubated for 5 min at 37°C and trypsin reaction was stopped with 100 µl ES-medium. 50 µl each were transferred to three 96-well plate (flat bottom) with feeder cells and incubated for three days. Two plates were frozen at -80°C (25 µl trypsin solution plus 25 µl FCS containing 20% DMSO) and the third one split on three gelatin-coated 96-well plates (flat
bottom). Cells were incubated until confluency, where two plates were washed twice with PBS and frozen at -20°C. The third plate was washed twice with PBS and incubated in ES-cell lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, 0.5% N-Lauroylsarcosine, 4% Proteinase K) over night at 56°C. DNA was precipitated by adding 100 µl isopropanol and 30 min incubation at RT. The supernatant was discarded and the pellet washed with 200 µl 70% EtOH, dried for 30 min at 37°C and resuspended in 25 µl TE plus RNAse A. 10 µl restriction digest mastermix (3.5 µl buffer, 2 µl enzyme, 0.01 µl 1 M DTT, 0.02 µl spermidine, 4.5 µl H₂O) were added and incubated over night at 37°C for Southern blot analysis (cf. 2.1.3).

**HTNC transduction**

Cre-mediated recombination *in vitro* was performed with a transducible His-TAT-NLS-Cre (HTNC) protein [Peitz *et al.*, 2002]. Cells were seeded on culture plates and grown to about 95% confluency. 1 µM HTNC was applied for 16-20 hours in DMEM / PBS [1:1] without antibiotics or supplements.

**Growth media**

Growth media were prepared under sterile conditions and stored at 4°C.

<table>
<thead>
<tr>
<th>Table 2.5: Cell Culture Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
</tr>
<tr>
<td>ES-medium</td>
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<td></td>
</tr>
<tr>
<td>EF-medium</td>
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</table>

Continued on next page
### Table 2.5 – continued from previous page

<table>
<thead>
<tr>
<th>Medium</th>
<th>Contents</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte-medium</td>
<td>DMEM with L-Glutamine</td>
<td>Gibco</td>
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<tr>
<td></td>
<td>10% FCS</td>
<td>Biochrom AG</td>
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<tr>
<td></td>
<td>1 mM Sodium Pyruvate</td>
<td>Gibco</td>
</tr>
<tr>
<td></td>
<td>1x Non-essential Aminoacids</td>
<td>Gibco</td>
</tr>
<tr>
<td></td>
<td>100 U/ml Pen-Strep</td>
<td>Gibco</td>
</tr>
<tr>
<td>Hepatocyte-fasting-medium</td>
<td>DMEM with L-Glutamine</td>
<td>Gibco</td>
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<tr>
<td></td>
<td>4% FCS</td>
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<tr>
<td></td>
<td>1x Non-essential Aminoacids</td>
<td>Gibco</td>
</tr>
<tr>
<td></td>
<td>100 U/ml Pen-Strep</td>
<td>Gibco</td>
</tr>
</tbody>
</table>

#### 2.1.3 Southern blot analysis

**Gel electrophoresis and blotting**

10-15 μg digested genomic DNA was loaded on an 0.8% agarose gel and run overnight at 30 V. The gel was incubated for 20 min in 0.25 M HCl for depurination. DNA was blotted on Hybond-XL™ charged nylon membrane (GE Healthcare, Freiburg, Germany) by alkaline capillary transfer with 0.4 M NaOH over night. The membrane was then washed for 20 min in 2x SSC (SSC: 300 mM NaCl, 30 mM Na$_3$C$_6$H$_5$O$_7$) buffer and cross-linked at 80°C for 40 min.

**Labeling and hybridization**

DNA fragments of the respective Southern probes were amplified by PCR or restriction digest (cf. table 2.6). Radioactive labeling with 25 μCi $\alpha^{32}$P-dCTP of 150 ng DNA was performed with the Ladderman DNA Labeling Kit (Takara Bio, Shiga, Japan). The membrane was incubated in 30 ml pre-hybridization solution (1 M NaCl, 50 mM Tris-HCl pH 7.5, 10% dextran sulfate, 1% SDS, 250 μg sonicated
salmon sperm DNA) for 4 h. The labeled probe was added and the membrane hybridized at 65°C over night. The membrane was washed in increasingly stringent Southern wash solution (2x SSC ↘ 0.5x SSC, plus 0.1% SDS) until the radioactive signal was below 30 mSv. Detection of the radioactive signal was done with Kodak MS hypersensitive films after over night exposure at -80°C.

### Table 2.6: Southern probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Source</th>
<th>Amplification</th>
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</thead>
<tbody>
<tr>
<td>SOCS3-U LA1</td>
<td>genomic DNA</td>
<td>5'-GATATCTCTGAATGCATCCAAGTTCTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GATATCGATGCACAGCCAGCTCTCA-3'</td>
</tr>
<tr>
<td>SOCS3-U RA1</td>
<td>genomic DNA</td>
<td>5'-GATATCACATGCTATGGCACACGTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GATATCTGGGATGAAGTTCCTGTC-3'</td>
</tr>
<tr>
<td>Neo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>A-04 plasmid&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5'-TGAATGAACTGCAGGAGGCA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GCCGCCAAGCTCTTCAGCAAT-3'</td>
</tr>
</tbody>
</table>

<sup>*</sup> Mao et al., 1999

### 2.2 Protein Biochemistry

#### 2.2.1 Protein extraction

Proteins from tissues or cells were extracted in organ lysis buffer (50 mM HEPES, 1% Triton X-100, 50 mM NaCl, 0.1 M NaF, 10 mM EDTA, 10 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1% SDS, 2 mM benzamidine, 10 µl/ml aprotinine, 2 mM PMSF, proteinase inhibitor cocktail, pH 7.4). Final protein lysates were diluted in organ lysis buffer and 4x SDS loading dye (250 mM Tris-HCL pH 6.8, 10% SDS, 87% glycerol, 200 mM DTT, 0.04% bromphenol blue).

**Protein extraction from tissues**

Organs were extracted from mice and a small part was incubated in 1 ml organ lysis buffer. The tissues were homogenized using an Ultra Turrax homogenizer (IKA, Staufen, Germany). The homogenate was centrifuged at 17,000 xg for 60 min at 4°C. Protein concentration of the cleared homogenate was determined using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.,
Materials and Methods

Schwerte, Germany) and adjusted to 10 µg/µl.

Protein extraction from cells

Cells were washed twice with PBS and scraped from the dish in an appropriate amount of organ lysis buffer. Cells were homogenized using a Vibrex VWR basic (IKA, Staufen, Germany) at 1000 rpm for 20 min at 4°C. The homogenate was centrifuged at 17,000 xg for 60 min at 4°C. Protein concentration of the cleared homogenate was determined using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Schwerte, Germany) and adjusted to 10 µg/µl.

2.2.2 Western blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Isolated proteins were separated by size via SDS-PAGE. Resolving gels contained 10% acrylamide if not stated otherwise. Gel electrophoresis was carried out in SDS running buffer (25 mM Tris, 200 mM glycine, 3.5 mM SDS) at 100 V for 2 h. 1.5 mm gels were used and loaded with PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Inc., Schwerte, Germany) and 100 µg protein per sample.

Western blot

Proteins were blotted from polyacrylamide gels onto PVDF-membrane (Bio-Rad, Munich, Germany) at 100 mA for 1 h. Membranes were blocked with 1% blocking reagent (Roche, Mannheim, Germany) in TBS-T for 1 h at RT. Incubation with the primary antibody in 0.5% blocking reagent was performed over night on a rotator at 4°C. Membranes were washed thrice in TBS-T for 10 min at RT and subsequently incubated with the appropriate secondary antibody in 0.5% blocking reagent for 1 h at RT on a rotator. Membranes were washed thrice in TBS-T for 10 min at RT and subsequently incubated in 10 ml Pierce ECL Western Blotting Substrate (Perbio Science, Bonn, Germany) for 1 min at RT. Detection of luminescence was performed.
Materials and Methods

using Amersham Hyperfilm chemiluminescent film (GE Healthcare, Little Chal-font, UK). Membrane stripping was done in stripping solution (65 mM Tris-HCl pH 6.8, 2% SDS, 0.7% β-Mercaptoethanol) for 30 min at 56°C if indicated. Table 2.7 contains a list of all antibodies used.

Table 2.7: Antibodies for Western blot

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cat.No.</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal anti-α-Tubulin</td>
<td>T6074</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>phospho-STAT3 (Y705) (D3A7) XP Rabbit mAb</td>
<td>9145</td>
<td>Cell-Signaling</td>
</tr>
<tr>
<td>SOCS3 Antibody</td>
<td>2923</td>
<td>Cell-Signaling</td>
</tr>
<tr>
<td>α-Rabbit IgG (whole molecule) Peroxidase Conjugate</td>
<td>A6154</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>α-Mouse IgG (whole molecule) Peroxidase Conjugate</td>
<td>A4416</td>
<td>Sigma-Aldrich</td>
</tr>
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</table>

2.3 Molecular Biology

2.3.1 quantitative Real-Time PCR

Quantitative Real-Time PCR was performed with the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems®, Foster City, USA).

mRNA isolation

mRNA from tissues and cells was isolated using the RNAeasy kit (Qiagen, Hilden, Germany). Tissues were homogenized using an Ultra Turrax homogenizer (IKA, Staufen, Germany), cells were homogenized with using QIAshredder columns (Qiagen). DNA was digested on-column with the RNase-Free DNase Set (Qiagen). Instructions given by the manufacturer were followed for all kits. RNA concentration was assessed by measuring absorption at 260 and 280 nm using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Schwerte, Germany) and adjusted to 200 ng/μl.

cDNA synthesis and qPCR setup

1 μg RNA was was reversely transcribed with High-Capacity cDNA Reverse
Transcription kit (Applied Biosystems, Foster City, USA) and amplified using TaqMan Gene Expression Master Mix with TaqMan Assay-on-demand kits (Applied Biosystems) following the instructions given by the manufacturer. Realtime probes used for gene expression analysis are listed in table 2.8. Relative expression was determined for target mRNA and samples were adjusted for total mRNA content by quantitative PCR for the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT). Calculations were performed by comparative method ($2^{-\Delta\Delta CT}$) [Livak & Schmittgen, 2001].

Table 2.8: Realtime Taqman probes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Transcript Name</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl20</td>
<td>Chemokine (C-C motif) ligand 20</td>
<td>Mm01268754_m1</td>
</tr>
<tr>
<td>Cd4</td>
<td>Cluster of differentiation 4</td>
<td>Mm00442754_m1</td>
</tr>
<tr>
<td>Cd8a</td>
<td>Cluster of differentiation 8</td>
<td>Mm01182107_g1</td>
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<td>Csf1</td>
<td>Colony stimulating factor 1</td>
<td>Mm00432685_m1</td>
</tr>
<tr>
<td>l17ra</td>
<td>Interleukin-17 receptor A</td>
<td>Mm00434214_m1</td>
</tr>
<tr>
<td>Il6ra</td>
<td>Interleukin-6 receptor a chain</td>
<td>Mm00439653_m1</td>
</tr>
<tr>
<td>Socs3</td>
<td>Suppressor of Cytokine Signaling 3</td>
<td>Mm00545913_s1</td>
</tr>
<tr>
<td>Tnfsf14</td>
<td>Tumor necrosis factor superfamily member 14</td>
<td>Mm00444567_m1</td>
</tr>
<tr>
<td>Hprt</td>
<td>Hypoxanthin-phosphoribosyl-transferase</td>
<td>Mm00446968_m1</td>
</tr>
</tbody>
</table>

2.3.2 Flow cytometry

Quantitative flow cytometry was performed either with the MACSQuant® VYB and MACSQuant® Analyzer 10 flow cytometers (both by Miltenyi Biotech, Bergisch Gladbach, Germany) or FACSCalibur™ flow cytometer (BD Bioscience, Heidelberg, Germany). Cell sorting was done with the FACSVantage™ SE cell sorters (both by BD Bioscience, Heidelberg, Germany).

Extracellular staining

Isolated non-parenchymal cells were treated with Trustain fcX™ for 15 min at 4°C. Cells were centrifuged at 400 xg for 5 min at 4°C. The pellet was resuspended in 100 µl FACS-buffer with Trustain fcX™ and the appropriate antibody for extracellular staining.
lular staining (cf. table 2.9). Cells were stained for 30 min at 4°C, and the reaction was stopped by adding 400 µl FACS-buffer. Cells were centrifuged at 400 xg for 5 min at 4°C and resuspended either in 200 µl FACS-buffer for analysis or 200 µl fix-perm buffer for intracellular staining.

**Intracellular staining**

Cells were incubated in fix-perm (#00-5123, eBioscience) for 30 min at RT and subsequently washed three times with 200 µl perm-buffer (#00-8333, eBioscience). Staining with the appropriate antibody (cf. table 2.9) was performed in 50 µl perm-buffer for 30 min at 4°C. Cells were washed twice in 200 µl perm-buffer and resuspended in 200 µl FACS-buffer for immediate analysis or IC-fix (#00-8222, eBioscience) for over night storage.

**Table 2.9: Antibodies for FACS**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cat.-No.</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trustain fcX™ (anti-mouse CD16/32)</td>
<td>101320</td>
<td>Biolegend</td>
</tr>
<tr>
<td>APC anti-mouse NK-1.1</td>
<td>108709</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Brilliant Violet 421™ anti-mouse CD3ε</td>
<td>100336</td>
<td>Biolegend</td>
</tr>
<tr>
<td>LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit</td>
<td>L34957</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>FITC anti-mouse NK-1.1</td>
<td>108705</td>
<td>Biolegend</td>
</tr>
<tr>
<td>PE/Cy7 anti-mouse F4/80</td>
<td>123114</td>
<td>Biolegend</td>
</tr>
<tr>
<td>PE/Cy7 anti-mouse TCR β</td>
<td>109222</td>
<td>Biolegend</td>
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<tr>
<td>PE anti mouse/rat CD126 (IL-6Ra chain)</td>
<td>115805</td>
<td>BioLegend</td>
</tr>
<tr>
<td>PE-CF594 anti-mouse CD3ε</td>
<td>562332</td>
<td>BD Horizon™</td>
</tr>
</tbody>
</table>

**Magnetic cell isolation and cell separation**

T-cells were separated from other non-parenchymal cells by MACS separation using CD90.2 microbeads (#130-049-101, Miltenyi Biotech) and LS columns (#130-042-401, Miltenyi Biotech). Instructions by the manufacturer were followed.

**Stimulation of Cytokine-production**

Cytokine production of isolated T-cells was stimulated *in vitro* prior to intra-
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cellular staining. T-cells were resuspended in 2 ml stimulation medium (DMEM, 10% FCS, 50 mg/ml PMA, 500 ng/ml Ionomycin, 1 μl/ml GolgiPlug™), seeded on a 6-well dish and incubated at 37°C for 6 h.

2.4 Animal handling

2.4.1 Animal care

Mice (Mus musculus, C57Bl/6N) were housed in a virus-free facility at 22-24°C on a 12 h light / 12 h dark cycle. Animals were fed standard rodent chow (Teklad Global Rodent 2018; 53.5% carbohydrates, 18.5% protein, 5.5% fat (12% calories from fat); Harlan, IN, USA). All animals had access to water and food ad libitum. Procedures and euthanasias were reviewed by the animal care committee, approved by local government authorities (Tierschutzkommission acc. §15 TSchG of the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen) and were in accordance with NIH guidelines.

2.4.2 Genotyping

Isolation of genomic DNA

Mouse tail biopsies for genotyping were taken at weaning age (D18-D25) and subsequently digested in 600 μl tail lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl) containing proteinase K (1/100) at 56°C over night. DNA was precipitated using 600 μl 100% isopropanol and centrifugation at 17,000 xg. Afterwards, DNA was washed with 500 μl 70% ethanol, centrifuged at 17,000 xg and dried. The DNA pellet was resuspended in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) containing RNase A (1/1000).

Polymerase chain reaction

For genotypic analysis, polymerase chain reaction (PCR) was performed on tail DNA using the primers given in table 2.10. For PCR DreamTaq PCR MasterMix and
DNA polymerase (Fermentas/Fisher Scientific Germany GmbH, Schwerte, Germany) was used. Standard PCR contained approx. 50 ng DNA, 25 pMol of each primer, 25 µM dNTP mix and 1 unit DNA polymerase in a 25 µl reaction mix.

Table 2.10: Genotyping Primer

<table>
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<tr>
<th>Mouse Line</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS3-U</td>
<td>5SOCS3U1</td>
<td>TTTTCTCTGGGGCGTCCTCCTAG</td>
</tr>
<tr>
<td></td>
<td>3SOCS3U2</td>
<td>AGCGCATCGGCTTCTGATGGCC</td>
</tr>
<tr>
<td></td>
<td>5SOCS3U3</td>
<td>CTGGATCTGACATGGTAAAGCTT</td>
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<td></td>
<td>3SOCS3U4</td>
<td>CCGCAGAGCGGCTGAGCTAC</td>
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<td></td>
<td>5SOCS3U5</td>
<td>CAAGCGGCTTCGCGGAGTAAC</td>
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<td>3SOCS3U6</td>
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<td></td>
<td>5SOCS3U7</td>
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<td>Alb-Dre</td>
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<td>Universal Dre</td>
<td>DreSeqJ</td>
<td>TGCTGTCTAGATCTGAGAGACT</td>
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<tr>
<td></td>
<td>Drerevneu</td>
<td>GCGGTGGTCTCCTCTAGAC</td>
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<tr>
<td>Universal Cre</td>
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<td></td>
<td>oIMR9105</td>
<td>CTTCTCGCTACGGCATGG</td>
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</table>

2.4.3 Mouse experiments

STAT3 signaling

STAT3 signaling was induced by i.p. injection of upstream ligands, where indicated, at the following concentrations: 50 ng/g IL-6, 20 ng/g TNFα, 250 ng/g LPS. Ligands were dissolved in PBS at a concentration corresponding to an injection volume of 10 µl/g.
DEN-injection

_long-term_ chronic Diethylnitrosamin (DEN) injection of 25 mg/kg BW [2.5 mg/ml H₂O] was performed in male mice _i.p._ 14 days after birth. Treated animals were sacrificed and their livers analyzed either 4 or 8 month post injection. _short-term_ acute DEN injection of 100 mg/kg BW [10 mg/ml H₂O] was performed in male mice _i.p._ 8-12 weeks after birth. Treated animals were sacrificed and their livers analyzed 0, 1, 2, 3 or 10 days post injection.

AOM/DSS treatment

Mice were injected _i.p._ with 10 mg/kg BW [1 mg/ml PBS] azoxymethane (AOM) and had their drinking water supplemented with 2.5% [w/v] dextran sulfate for 5 days. Treated animals received pure drinking water for additional 3 days before they were sacrificed and their colons analyzed.

Isolation and cell culture of primary hepatocytes

Mice were anesthetized and their liver perfused via vena cava with EBSS-perfusion buffer (0.5 mM EGTA in EBSS) for 5 min, followed by perfusion with 50 ml EBSS-collagenase buffer (10 mM HEPES, 15 mg Collagenase IV, 2 mg trypsin inhibitor in EBSS). Afterwards, hepatocytes were released from cell association in 10 ml EBSS and filtered through a 100 μm strainer. Cells were washed twice with hepatocyte culture medium (Table 2.5). Cells were counted and an appropriate cell number, depending on the respective growth area, was seeded on collagen coated culture plates (BD Biocoat). Culture medium was changed after 4 hours. Cells were kept in hepatocyte-medium for another 12 hours and then starved in hepatocyte-fasting-medium for 4 hours. Hepatocytes were stimulated with 50 ng/ml IL-6 in hepatocyte-fasting-medium for the indicated timepoints.

Isolation of non-parenchymal liver cells

Mice were sacrificed and their liver perfused with HBSS via vena cava. Liv-
ers were incubated in 5 ml liver dissociation buffer (150 mM NaCl, 5.6 mM KCl, 5.5 mM Glucose, 20.1 mM HEPES, 25 mM NaHCO3, 2 mM CaCl2, 2 mM MgCl2, 500 U/ml collagenase IV, 150 U/ml DNAse I, pH 7.4), disrupted with the gentleMACS™ dissociator (Miltenyi Biotech, Bergisch Gladbach, Germany) and further incubated at 37°C. Cells were filtered through a 100 µm strainer, and both the c-tube as well as the strainer washed with PEB (0.5% BSA, 2 mM EDTA in PBS). The filtrate was centrifuged at 50 xg, 4°C for 5 min and the supernatant, containing the NPC fraction, filtered through a 40 µm strainer. The hepatocyte pellet was resuspended in 30 ml PEB, again centrifuged and the supernatant also filtered through the 40 µm strainer. The filtrate was centrifuged at 350 xg, 4°C for 10 min and the NPC-pellet resuspended in PEB up to a volume of 5 ml.

The NPC solution was mixed with 5 ml 40% Histodenz [w/v PBS] and 5 ml each underlayed 5 ml PEB in a small falcon tube for gradient purification. The gradients were centrifuged at 1500 xg, 4°C for 20 min without brake and the NPCs collected from the interface. NPCs from both tubes were combined and diluted in PEB up to a volume of 50 ml. Cells were centrifuged at 350 xg, 4°C for 10 min and the NPC-pellet resuspended in FACS buffer (2% FCS, 2 mM EDTA in PBS).

2.5 Chemicals and Materials

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-mercaptoethanol</td>
<td>Applichem, Darmstadt, Germany</td>
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<td>Acrylamide</td>
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<tr>
<td>Agarose</td>
<td>Peqlab, Erlangen, Germany</td>
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<tr>
<td>Ammoniumpersulfat (APS)</td>
<td>Sigma-Aldrich, Seelze, Germany</td>
</tr>
<tr>
<td>Bacillol</td>
<td>Bode Chemie, Hamburg, Germany</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma-Aldrich, Seelze, Germany</td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Desoxy-ribonucleotid-triphosphates (dNTPs)</td>
<td>Amersham, Freiburg, Germany</td>
</tr>
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<td>Developer G153</td>
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<td>DEPC</td>
<td>Applichem, Darmstadt, Germany</td>
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</table>

Continued on next page
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylsulfoxide (DMSO)</td>
<td>Merck, Darmstadt, Germany</td>
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<tr>
<td>Enhanced chemiluminescence (ECL) Kit</td>
<td>Perbio Science, Bonn, Germany</td>
</tr>
<tr>
<td>Ethanol, absolute</td>
<td>Applichem, Darmstadt, Germany</td>
</tr>
<tr>
<td>Ethidium bromide</td>
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</tr>
<tr>
<td>Ethylendiamine tetraacetate (EDTA)</td>
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<td>Formamide</td>
<td>Applichem, Darmstadt, Germany</td>
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<td>Glycerol</td>
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<td>GolgiPlug™</td>
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<td>Hydrochloric acid (37%)</td>
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<td>Isopropanol (2-propanol)</td>
<td>Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>LB</td>
<td>Applichem</td>
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<tr>
<td>Magnesium chloride</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Methanol</td>
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<tr>
<td>Nitrogen (liquid)</td>
<td>Linde, Pullach, Germany</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma-Aldrich, Seelze, Germany</td>
</tr>
<tr>
<td>Phenol/Chloroform/Isoamylalkohol</td>
<td>Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>Gibco BRL, Eggenstein, Germany</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Potassium dihydrogenphosphat</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Potassium hydroxide</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>Applichem, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium chloride</td>
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3 Results

Inflammatory signaling comprises a plethora of metabolic processes. Its main function lies in the defense against infections, but the immune system tries also to counteract non-infectious burdens on the organism. The development of cancer heavily depends on the infiltration of immune cells into the tumor microenvironment. In case of so called inflammation-driven cancers, the secreted immune factors, such as cytokines and chemokines, are even a prerequisite for the tumor development. A low grade chronic inflammation has also been observed under obese conditions, where white adipose tissue stress in turn attract immune cells to this site.

3.1 Generation of a universal reporter tool for inflammatory signaling

The suppressor of cytokine signaling 3 (SOCS3) gene is part of a negative feedback-loop for numerous intracellular pathways, most prominently the JAK/STAT pathway or NF-κB, and is strongly upregulated independent of the particular cause of inflammation. Therefore the SOCS3 locus has been chosen to drive the SOCS3-U allele, a reporter tool to visualize active inflammatory signaling.

3.1.1 Genetic features of SOCS3-U

The SOCS3 Universal Tumormicroenvironment Activation Measurement Allele (SOCS3-UnTAMAble / SOCS3-U) is a versatile reporter tool to measure inflammatory signaling in vitro and in vivo. The activity of the allele is controlled by a loxP-flanked neo-stop cassette and depends on Cre-mediated recombination,
The SOCS3-U allele is controlled by a loxP-flanked neo-stop cassette. The rox-flanked endogenous SOCS3 CDS can be excised by Dre, and the IRES-GFP reporter can be switched to firefly luciferase expression by FLP-mediated recombination.

which not only terminated transcription, but also serves as a survival signal as it provides resistance against G418. Once active, the reporter allele utilizes the endogenous SOCS3 promoter to drive compound expression of SOCS3 and IRES-GFP. The SOCS3 coding sequence (CDS) is flanked by rox-sites and can be excised by Dre-mediated recombination, yielding a functional knock-out of SOCS3 in Dre-expressing tissues. After Dre-mediated recombination, the reporter allele can be modified by FLP-mediated recombination to switch from IRES-GFP to express firefly luciferase, enabling the visualization of SOCS3 activity in vivo in a non-invasive manner (Fig. 3.1).

3.1.2 SOCS3-U targeting vector generation

For the generation of the SOCS3-U targeting vector, existing genetic tools available in our lab could be used. The common Stop-eGFP-ROSA-CAGs [SERCA, Klisch, 2006] plasmid contains a loxP-flanked neo-stop cassette, followed by an IRES-GFP reporter cassette flanked by FRT sites in tandem orientation. In a first step, SOCS3 CDS was amplified by PCR from genomic DNA. Rox-sites for Dre-mediated recombination are small enough to be introduced into the PCR amplicon as primer overhangs. The rox-SOCS3-rox amplicon was digested with MluI restriction enzyme and ligated between the loxP-flanked stop cassette and the IRES-GFP reporter cassette into the Ascl-digested SERCA plasmid. The complete loxP-neo/stop-loxP-rox-SOCS3-rox-frt-IRES-GFP-frt fragment was subsequently excised from the SERCA backbone with an NheI/EcoRV double digest and ligated into the GK12TK plasmid () backbone, cut with AvrII and Pmel. The left arm of homology
(LAH), necessary for targeting of the SOCS3 locus, was amplified by PCR from genomic DNA, digested with PvuI and PacI and inserted into the PacI restriction site in front of the loxP-flanked neo/stop cassette. The right arm of homology (RAH) for the SOCS3 was amplified by PCR from genomic DNA and the firefly luciferase CDS was amplified via PCR from the pTE-Luc plasmid. The luciferase amplicon contained a FRT site at the 5’ end, introduced as a primer overhang, and was ligated as a reverse complement in front of the RAH into the pGEM-T-Easy cloning vector with AscI/SwaI double digest. The resulting luciferase-frt–RAH fragment was cut from the pGEM-T-Easy backbone via AgeI restriction digest and ligated at the 3’ end of targeting construct into the GK12TK plasmid.

The SOCS3-U allele was targeted into the SOCS3 locus, which consists of two exons. Exon 1 and the first 88 nucleotides of exon 2 form the 5’UTR. The coding sequence of SOCS3 is located within exon 2, followed by a 3’UTR (Fig. 3.2a). A targeting vector flanked by 5 kb homologous regions (LAH and RAH, respectively) was constructed, ensuring the correct targeting to the endogenous SOCS3 locus. To prevent random integration of the construct into the mouse genome, the targeting construct contains the negative selection marker herpes-simplex-virus thymidine kinase (HSV Tk) cassette besides the homologous regions (Fig. 3.2b). Upon random integration of HSK Tk, cells will also contain and express the viral thymidine kinase, which renders them susceptible to ganciclovir treatment. Correctly targeted cells only contain the region between the homologous regions and are therefore resistant to G418 and ganciclovir. Southern blot analysis can be performed to identify correctly targeted ES cells. Integration of the SOCS3-U allele introduces additional EcoRV restriction sites, yielding two smaller fragments (9 & 9.7 kb for probes LA1 and RA1 respectively) compared to the 17.9 kb wt fragment (Fig. 3.2c).

3.1.3 Successful targeting of the endogenous SOCS3 locus

10⁷ Bruce4 C57BL/6 ES cells [Köntgen et al., 1993] were transfected with 40 µg AsiSI-linearized SOCS3-U targeting construct and subsequently selected for G418
Figure 3.2: SOCS3-U targeting construct is targeted to the murine SOCS3 locus
(a) The murine SOCS3 locus comprises 2 exons, with exon 1 and the first
88 nucleotides of exon 2 forming the 5'UTR, and a 1.5 kb 3'UTR. The coding
sequence, depicted in black, lies completely within exon 2. (b) Correct targeting
of the targeting construct was assured by 5 kb homologous regions with the
targeted locus (LAH and RAH respectively). The targeting construct contains a
loxP flanked neo-stop cassette, the rox-flanked endogenous SOCS3 exon 2 and
a FRT-flanked IRES-driven eGFP / firefly luciferase double reporter. Random,
non-homologous recombination of the construct was inhibited by the HSV TK
cassette outside the homologous regions of the targeting construct. (c) Integration
of the targeting construct into the murine SOCS3 locus could be verified
by Southern blot analysis. Integration of the targeting construct introduces ad-
ditional EcoRV restriction sites, yielding two smaller fragments (9 & 9.7 kb for
probes LA1 and RA1 respectively) compared to the 17.9 kb wt fragment, or a
6.1 kb fragment for the neo-probe, hybridizing with the neomycin resistance
cassette.

resistance for 10 days. 800 ES cell clones were isolated, expanded and analyzed
for correct integration of the SOCS3-U knock-in allele into the endogenous SOCS3
locus via Southern blot analysis. Southern-blot analyses of EcoRV-digested clonal
dNA using both LA1 and RA1 probes on ES cell clones identified 3H2 and 8A10 as
these clones showed the expected transgenic bands (9 & 9.7 kb for probes LA1 and
RA1 respectively) besides the 17.9 kb wt band, confirming successful, heterozygous
targeting of ES cells with the SOCS3-U targeting construct (not shown). Correctly targeted 3H2 and 8A10 ES-cell clones were expanded and subjected to in depth Southern-Blot analysis, confirming that both clones show the expected, additional 9 & 9.7 kb bands (Fig. 3.3). ES cells from the 3H2 and 8A10 clones were injected into C.B20 blastocysts to obtain chimeras of SOCS3-U^{fl;rox;frt-GFP/wt} mice. 3 chimeras were derived from the 3H2 clone, but displayed only very weak chimerism (5%, 5% and 20%), while 4 chimeras derived from the 8A10 clone exhibited higher chimerism (50%, 55%, 75% and 85%). Only the 75% chimera derived from the 8A10 clone transmitted the SOCS3-U allele through the germline, successfully establishing the SOCS3-U mouse line.

Collectively, we have successfully generated the SOCS3-U targeting vector, targeted ES-cells and SOCS3-U mice.

3.2 SOCS3-U variants to analyze inflammatory signaling

The SOCS3-U allele is present in three variants for analytic purposes. The parental SOCS3-U^{fl;rox;frt-GFP} variant represents the inactivated state. This allele functions as a knock-out-first allele, since expression of the endogenous SOCS3 CDS is inhibited by the floxed neo-stop cassette (Fig. 3.4a). Since a whole-body
Figure 3.4: SOCS3-U variants before and after site-specific recombination

(a) The basic SOCS3-U^{fl;rox;frt-GFP} allele does neither express SOCS3, nor any of the reporter genes. (b) After Cre-mediated recombination, SOCS3-U^{Δ;rox;frt-GFP} expresses SOCS3 and an IRES driven GFP in the given target tissues. (c) Via Dre- and FLP-mediated recombination, the SOCS3-U^{fl;Δ;frt-Luc} allele does no longer express SOCS3, but utilizes the SOCS3 promoter to drive expression of firefly luciferase. The allele still has to be activated by Cre-mediated recombination.

SOCS3 ablation is embryonic lethal [Roberts et al., 2001], the basic allele can only be kept heterozygous in SOCS3-U^{fl;rox;frt-GFP/wt} mice. Cre-mediated recombination will yield the SOCS3-U^{Δ;rox;frt-GFP} variant and re-enable the expression of SOCS3, as well as of IRES-GFP (Fig. 3.4b). Homozygous SOCS3-U^{Δ;rox;frt-GFP/Δ;rox;frt-GFP} mice can therefore be crossed to Dre-driver lines, thus generating tissue-specific SOCS3 knock-outs. For non-invasive in vivo imaging, FLP-mediated recombination can be used on Dre-deleted SOCS3-U^{fl;Δ;FRT-GFP} allele to generate the SOCS3-U^{fl;Δ;frt-Luc} variant (Fig. 3.4c). After subsequent Cre-mediated activation, firefly luciferase can be expressed from the endogenous SOCS3 promoter. D-luciferin can be injected i.p. and will be oxidized by luciferase in an ATP-dependent manner. The invested energy is released as bioluminescence upon decay of the formerly oxidized product. Thus, these SOCS3-U variants will help to characterize inflammatory signaling in the tumor microenvironment in different ways.
3.3 SOCS3-U can successfully be utilized *in vivo*

With generation of SOCS3-U mice, the ability to be activated *in vivo* and an exclusion of a potential phenotype of unactivated SOCS3-U^fl;rox;frt-GFP/wt^ mice had to be investigated.

### 3.3.1 Cre-activated SOCS3-U cells express GFP upon stimulation

In order to assess the ability of SOCS3-U to express GFP upon stimulation of SOCS3, SOCS3-U 8A10 ES cells were transfected with pPGK-Cre-bpA plasmid [Kurt Fellenberg, University of Cologne]. 200 clones were picked and successful Cre-mediated excision of the loxP-flanked stop cassette was assessed by Southern blot analyses. ES cell clone H2 contained the Cre-excised SOCS3-U allele (Fig. 3.5b). H2 cells were expanded and stimulated with LIF, LPS, or IL-6 for 24h. FACS quantification of stimulated SOCS3-U ES cells confirmed SOCS3 dependent GFP activity upon stimulation, though to a low extent (Fig. 3.5c).

To verify whether SOCS3-U can be activated by Cre-mediated recombination *in vivo*, SOCS3-U^fl;rox;frt-GFP/wt^ mice were crossed to Cre-deleter mice [Schwenk *et al.*, 1995] in order to activate SOCS3-U in the whole body (SOCS3-U^Δ;rox;FRT-GFP/wt^). Peritoneal macrophages and splenocytes were isolated and stimulated *ex vivo* with LPS, IL-6, IL-11 or TNFα for 24h. Stimulated macrophages exhibit increasing GFP fluorescence with IL-6, IL-11 and TNFα compared to LPS-stimulated cells or unstimulated macrophages (Fig. 3.6a). In contrast, splenocytes display similar levels of enhanced GFP fluorescence upon stimulation with LPS, IL-11, TNFα and IL-6 when compared to unstimulated splenocytes (Fig. 3.6b).

Taken together, these experiments demonstrate that the SOCS3-U allele works *in vitro* and *ex vivo*, though to minor effect under these conditions *in vivo*.

### 3.3.2 Wild-type like inflammation in SOCS3-U^fl;rox;frt-GFP/wt^ mice

The SOCS3-U allele is conditionally activated to utilize the GFP or luciferase reporter as specifically as possible, i.e. either with spatial or temporal con-
Figure 3.5: GFP fluorescence of SOCS3-U ES cells after Cre activation
(a) Genomic DNA of transfected 8A10 ES cells was isolated and digested with NsiI and analyzed with the RA1 probe, yielding an 11.3 kb fragment for the wt SOCS3 allele, a 17.9 kb fragment for the targeted SOCS3-U allele and an 15.5 kb fragment after Cre-mediated activation. (b) ES cell clone H2 shows the correct 15.5 kb band for Cre-mediated active SOCS3-U. (c) Stimulation with LIF [4x10^4 U], LPS [500 ng/ml] and IL-6 [30 ng/ml] upregulated GFP expression compared to unstimulated SOCS3-U control cells.

control or a combination of both. Hence, off-target tissues will retain the basic SOCS3-U<sup>flox;frt-GFP</sup> allele, rendering them heterozygous SOCS3 knock-outs. In order to address, whether a heterozygous SOCS3 knock-out affects the duration or severity or inflammatory signaling, primary hepatocytes from either wt or SOCS3-U<sup>flox;frt-GFP/wt</sup> mice were isolated and 2x10<sup>5</sup> stimulated in vitro with 50 ng/ml IL-6 for 0, 15, 30, 60 or 120 min. IL-6 signaling both in wt and SOCS3-U<sup>flox;frt-GFP/wt</sup> mice leads to phosphorylation of STAT3 (pSTAT3) already after 15 min (Fig. 3.7a). SOCS3 protein levels begin to increase 30 min after IL-6...
stimulation, where also the highest pSTAT3 levels are detected. SOCS3 activity subsequently blocks IL-6 downstream signaling, consistently decreasing pSTAT3 levels 60 and 120 min after stimulation. Furthermore, Analyzation of SOCS3 mRNA expression 0, 15 and 120 min after IL-6 stimulation from isolated control as well as SOCS3-\(U^{\Delta\text{rox};\text{FRT}-\text{GFP}/\text{wt}}\) primary hepatocyte RNA showed no differences in SOCS3 mRNA upregulation between wt and SOCS3-\(U^{\Delta\text{rox};\text{FRT}-\text{GFP}}\) primary hepatocytes (Fig. 3.7b).

Importantly, a heterozygous SOCS3 knock-out in primary hepatocytes from SOCS3-\(U^{\Delta\text{rox};\text{FRT}-\text{GFP}/\text{wt}}\) mice has no effect on the dynamics of IL-6 signaling in vitro. Thus, utilization of SOCS3-\(U^{\Delta\text{rox};\text{FRT}-\text{GFP}/\text{wt}}\) mice in combination with tissue-specific Cre-driver lines to active SOCS3-U has no detrimental effect on inflammatory signaling, at least in hepatocytes, and can be used as GFP or luciferase reporters. However, no SOCS3-\(U^{\Delta\text{rox};\text{FRT}-\text{Luc}/\text{wt}}\) animals to analyze SOCS3-U driven luciferase expression could be obtained so far.

3.4 Contribution of macrophages to AOM/DSS induced CRC

Colorectal cancer (CRC) formation is a classic case of inflammation associated
3.4.1 IL-6 mediated colorectal cancer formation

Previous data from our lab indicate that IL-6Rα ablation has a protective effect on colorectal cancer formation, in that IL-6Rα deficient mice (IL-6Rα^\Delta/\Delta) exhibit reduced AOM/DSS induced tumors [Claudia Wunderlich, University of Cologne]. A microarray analyses of these IL-6Rα deficient vs. proficient tumors revealed decreased levels of CCL20 (chemokine ligand 20), CCR6 (chemokine receptor 6) and various lymphocyte markers in IL-6Rα^\Delta/\Delta tumors. CCL20 is a lymphocyte attracting chemokine [Hieshima et al., 1997] and signals via CCR6 on target cells [Baba et al., 1997]. This observation suggests an IL-6 dependent mechanism of CCL20 expression, attracting lymphocytes to the colon to promote colorectal cancer.
Abrogation of IL-6 signaling in myeloid cells severely diminishes their capability of differentiating into alternatively activated towards M2 macrophages [Mauer et al., 2014]. As IL-6RaΔ/Δ mice exhibit no M2 macrophages and reduced CCL20 expression, this led to the hypothesis that M2 macrophages during colitis attract lymphocytes to the colon, thereby promoting colorectal cancer formation. In order to shed light on the macrophage subpopulations and their contribution to colorectal cancerogenesis, SOCS3-Ufl;rox;frt-GFP/wt mice were crossed to LysM-Cre<sup>tg</sup>/wt mice [Clausen et al., 1999]. Furthermore, double-positives were subsequently crossed to R26-fl-tdTomato<sup>fl/fl</sup> mice [Madisen et al., 2010] to control for Cre-mediated recombination by red fluorescence. The resulting SOCS3-Ufl;rox;frt-GFP/wt;R26-fl-tdTomato<sup>fl/fl</sup>;LysM-Cre<sup>tg</sup>/wt mice were subjected to AOM/DSS treatment to recapitulate the early stage of acute colitis as a driving force for CRC development. SOCS3-Ufl;rox;frt-GFP/wt;R26-fl-tdTomato<sup>fl/fl</sup>;LysM-Cre<sup>tg</sup>/wt mice will express the red fluorescent tdTomato protein in all myeloid lineage derived cells, and express GFP in a SOCS3 dependent manner.

3.4.2 SOCS3<sup>low</sup> M2 macrophages express CCL20 and IL-17RA

After AOM injection, 5 days of DSS treatment and 3 days recovery phase, macrophages were isolated from the colon and subjected to fluorescence activated cell sorting. Untreated control animals exhibit a small cohort of red fluorescent tdTomato<sup>high</sup>;GFP<sup>low</sup> macrophages, and virtually no tdTomato<sup>high</sup>;GFP<sup>high</sup> macrophages (Fig. 3.8a). AOM/DSS treatment strongly increases the amount of macrophages in the colon, both of the tdTomato<sup>high</sup>;GFP<sup>low</sup> type as well as tdTomato<sup>high</sup>;GFP<sup>high</sup> macrophages (Fig. 3.8b). To investigate which if those macrophages express CCL20, RNA from sorted macrophages was isolated and the expression level of CCL20 mRNA was analyzed by qPCR (Fig. 3.8c). Expression of CCL20 in tdTomato<sup>high</sup>;GFP<sup>low</sup> macrophages upon AOM/DSS treatment is significantly upregulated 6-fold compared to untreated tdTomato<sup>high</sup>;GFP<sup>low</sup>
Results

Figure 3.8: SOCS3\textsuperscript{low} M2 macrophages express CCL20 upon AOM/DSS treatment
Representative FACS blots of macrophages isolated from colons of SOCS3-\textsuperscript{L\textsubscript{L}f\textsuperscript{lox}\textsuperscript{frt}\textsuperscript{-GFP}\textsuperscript{int};R26-fl-t\textsuperscript{tdTomato}\textsuperscript{fl};\textsuperscript{LysM-Cre}\textsuperscript{fl} mice either (a) untreated or (b) treated with AOM/DSS for 5 days. Macrophages were sorted for td\textsuperscript{Tomato\textsuperscript{high};GFP\textsuperscript{low}} (red square) and td\textsuperscript{Tomato\textsuperscript{high};GFP\textsuperscript{high}} (green square) subpopulations. RNA from sorted cells was isolated and the expression level of (c) CCL20, (d) IL-17RA and (e) IL-6R\textsubscript{\alpha} mRNA was analyzed by qPCR. Displayed are means ± SEM, n=4. * p ≤ 0.05

macrophages. Moreover, CCL20 expression is also significantly higher than in td\textsuperscript{Tomato\textsuperscript{high};GFP\textsuperscript{high}} macrophages, which exhibit only a 2.5 fold upregulation from the untreated controls. Furthermore, td\textsuperscript{Tomato\textsuperscript{high};GFP\textsuperscript{low}} macrophages have 4-fold upregulated IL-17 receptor (IL-17RA) expression compared to untreated macrophages (Fig. 3.8d). IL-17 producing T\textsubscript{H}17 cells are known to massively infiltrate inflamed intestines [Gálvez, 2014]. Lastly, only td\textsuperscript{Tomato\textsuperscript{high};GFP\textsuperscript{low}} macrophages express IL-6R\textsubscript{\alpha} and not td\textsuperscript{Tomato\textsuperscript{high};GFP\textsuperscript{high}} macrophages (Fig. 3.8e). Cell numbers of untreated td\textsuperscript{Tomato\textsuperscript{high};GFP\textsuperscript{high}} macrophages were too low to analyze mRNA expression.

Taken together, SOCS3\textsuperscript{low} M2-like tumor associated macrophages (TAMs) express significantly higher levels of CCL20 and IL-17RA upon AOM/DSS treatment than SOCS3\textsuperscript{high} M1-like macrophages. Additionally, SOCS3\textsuperscript{high} M1-like macrophages do not express IL-6R\textsubscript{\alpha}, consistent with the CCL20 expression profile upon IL-6R\textsubscript{\alpha} ablation. These date confirm a distinct influence of M2 TAMs on CRC de-
development and raise the possibility of an IL-17 mediated activity upon increased inflammation in the colon.

3.5 Investigating macrophage subpopulations in DEN-induced HCC

Hepatocellular carcinoma development is a classical inflammation associated cancer that can be experimentally elicited by injection of diethylnitrosamin (DEN) into 12-15 days old male mice [Vesselinovitch & Mihailovich, 1983]; DEN treated mice show hepatocellular carcinoma after 8 months.

The impact of Kupffer cell derived cytokines on tumor progression has been suggested but still remains to be proven [Naugler et al., 2007]. SOCS3-U^fl;rox;frt-GFP/wt;R26-fl-tbTomato^fl/wt;LysM-Cre^tg/wt mice were subjected to the chronic DEN HCC model, in order to elucidate the contribution of the various macrophage subpopulations, namely liver resident Kupffer cells, classically activated M1 macrophages and alternatively activated M2 macrophages, to hepatocellular carcinogenesis. SOCS3-U^fl;rox;frt-GFP/wt;R26-fl-tbTomato^fl/wt;LysM-Cre^tg/wt mice will express the red fluorescent tdTomato protein in all myeloid lineage derived cells, and additionally express GFP in a SOCS3 dependent manner. Kupffer cells are not affected by LysM-Cre [Hume, 2011] and therefore express neither tdTomato nor GFP, but classic macrophage markers such as F4/80 [Austyn & Gordon, 1981].

SOCS3-U^fl;rox;frt-GFP/wt;R26-fl-tbTomato^fl/wt;LysM-Cre^tg/wt mice were sacrificed after 8 months and non-parenchymal liver cells were isolated and labeled with an α-F4/80-PE/Cy7 antibody. Fluorescently labeled cells could be sorted into three distinct subpopulations, namely tdTomato^{low},F4/80^{high} Kupffer cells, tdTomato^{high},GFP^{low} alternatively activated macrophages and tdTomato^{high},GFP^{high} classically activated macrophages (Fig. 3.9a,b). Determination of the cell numbers revealed around 30% of all non-parenchymal liver cells to be tdTomato expressing macrophages and approximately 5% be-
Results

Figure 3.9: SOCS3-U labels activated macrophages in DEN-induced HCC

Representative FACS blots of non-parenchymal liver cells isolated from three SOCS3-U\textsuperscript{fl;rox;frt-GFP/wt;R26-fl-tdTomato\textsuperscript{fl;lat};LysM-Cre\textsuperscript{tg/wt}} mice, labeled with an \(\alpha\)-F4/80-PE/Cy7 antibody. (a) tdTomato\textsuperscript{low};F4/80\textsuperscript{high} cells were sorted from tdTomato\textsuperscript{high};F4/80\textsuperscript{high} cells. (b) Cells were further divided into SOCS3-U dependent GFP\textsuperscript{high} and GFP\textsuperscript{low} cells. Quantification of cell numbers for tdTomato\textsuperscript{high};GFP\textsuperscript{low}, tdTomato\textsuperscript{high};GFP\textsuperscript{high} and tdTomato\textsuperscript{low};F4/80\textsuperscript{high} cells in (c) absolute cell numbers sorted or as (d) relative cell counts of all non-parenchymal cells in percent. Displayed are means ± SEM, n=8 for (c) and n=3 for (d).

ing Kupffer cells (Fig. 3.9d). On average, around 4.8x10\(^4\) of macrophages sorted were tdTomato\textsuperscript{high};GFP\textsuperscript{low} alternatively activated macrophages, 1.2x10\(^4\) were tdTomato\textsuperscript{high};GFP\textsuperscript{high} classically activated macrophages and 5x10\(^3\) were tdTomato\textsuperscript{low};F4/80\textsuperscript{high} Kupffer cells (Fig. 3.9c).

Collectively, application of SOCS3-U\textsuperscript{fl;rox;frt-GFP/wt;R26-fl-tdTomato\textsuperscript{fl;lat};LysM-Cre\textsuperscript{tg/wt}}
mice in the DEN-induced HCC model enables the separation of infiltrating M1 macrophages from M2 macrophages as well as Kupffer cells. These respective macrophage subpopulations can then be subjected to further in depth analysis, e.g. transcriptome sequencing, to address their contribution to DEN-induced HCC development.

3.6 Activation of T-cells in the tumor microenvironment of HCC

Hepatocellular carcinoma is one of the most common cancers and has a very high mortality rate of around 95%. HCC develops predominantly from cirrhosis after hepatitis virus B or C infection, but it is also strongly driven by excessive alcohol consumption or non-alcoholic steatohepatitis (NASH). Similar to a hepatic viral infection, NASH causes enhanced inflammation in the liver, which is the common driver for HCC initiation. Consistently, infiltration and activation of immune cells into the steatotic liver could be observed in a mouse model of a choline deficient high fat diet (CD-HFD), recapitulating clinical observations of choline deficiency in NASH patients [Wolf et al., 2014]. Furthermore, hepatic lipid uptake, and ultimately the transition from NASH to HCC, could be linked to LIGHT (TNFsf14, tumor necrosis factor ligand superfamily member 14) secretion from NK-T cells.

3.6.1 IL-6 dependent modification of LIGHT expression

Our previous data demonstrated that the protective effect of a whole body IL-6Rα deficiency (IL-6RαΔ/Δ) was abrogated under obese conditions [Gruber et al., 2013]. Interestingly, IL-6Rα deficiency specifically in T-cells (IL-6RαT-KO) protects from HCC development in lean animals and even during obesity [Gruber, 2013]. Furthermore, IL-6Rα deficiency in hepatocytes showed unaltered HCC development, indicating that IL-6 signaling on T-cells is critical for HCC development. Together with the experiments by Wolf et al., we hypothesize that IL-6 induces LIGHT ex-
expression to promote hepatic lipid uptake and progression from NASH to HCC, corroborated by an analysis of the LIGHT promoter, revealing 30 STAT regulatory binding sites 5000 bp upstream of the transcription start (Fig. 3.10a). To investigate this, control mice were injected with IL-6 [50 ng/g] i.p. and hepatic LIGHT expression was analyzed after 30, 60, 120 and 240 minutes in order to address, whether expression of LIGHT is regulated by IL-6 signaling. Stimulation with IL-6 leads to a 1.5 fold induction of hepatic LIGHT mRNA expression already after 30 min, reaching significance after 60 min with a 2.5 fold induction and peaking with a 3-fold upregulation after 2h (Fig. 3.10b). This clearly demonstrates that LIGHT mRNA expression in the liver is affected by IL-6 signaling.

To shed light on the contribution of IL-6 signaling on hepatic LIGHT mRNA expression in the tumor initiating phase of DEN-induced HCC development, $IL-6R^{α/β}$, $IL-6R^{Δ/Δ}$ and $IL-6R^{T-KO}$ mice were subjected to the acute DEN model and LIGHT mRNA expression was analyzed after 1, 2, 3 and 10 days in whole-liver extracts by qPCR. It was already demonstrated that acute DEN treatment
stimulates IL-6 release into the blood stream, peaking after 8 hours with a 4 fold concentration and returning to baseline levels after 30 hours [Gruber et al. 2013]. Acute DEN treatment leads to significant LIGHT mRNA downregulation after 24h both in IL-6Rαfl/fl and IL-6RαΔ/Δ animals as well as IL-6RαT-KO mice, persisting until 48h after DEN injection (Fig. 3.11). Subsequently, LIGHT mRNA expression is significantly increased 72h after DEN treatment, with a 2 fold upregulation compared to the basal state in IL-6Rαfl/fl and IL-6RαT-KO mice and up to 3 fold in IL-6RαΔ/Δ mice. LIGHT mRNA expression continues to be elevated and is still upregulated 1.5-2 fold in all genotypes after 10 days. Collectively, IL-6 signaling has an inhibitory effect on hepatic LIGHT mRNA expression in the first 48h after DEN injection, recapitulating the early, tumor initiating phase of hepatocellular carcinogenesis, followed by a significant induction of LIGHT mRNA expression.

Taken together, LIGHT expression is clearly affected by IL-6 signaling and altered upon high-dose DEN injection, but with minor differences between presence or absence of IL-6Ra, both in all cell types or only in T-cells. In line with these experiments, Wolf et al. show that LIGHT in CD-HFD induced HCC was derived from NK-T cells, which are a special T-cell subpopulation. Furthermore, we have demonstrated earlier that only a marginal population of NK-T cells (i.e. 2%), the source of LIGHT expression, do express IL-6Ra [Gruber 2013]. However, with uti-
Figure 3.12: **Acute DEN elevates SOCS3-GFP expression**

GFP fluorescence histograms of non-parenchymal liver cells isolated from SOCS3-\(\text{U}^{\text{fl;rox;fert-GFP/wt}}\),\(\text{CD4-Cre}\text{tg/wt}\) mice. SOCS3-GFP intensity is displayed (a) 1 day after acute DEN treatment, (b) 2 days after treatment or (c) 3 days after treatment compared to untreated control animals in gray.

Ligation of SOCS3-U reporter mice, active cell-type specific IL-6 signaling can be visualized to gain further insight into the contribution of IL-6 signaling on NK-T cells with respect to LIGHT mRNA expression.

### 3.6.2 Separation of NK-T cell subpopulations using SOCS3-U

In order to address, whether NK-T cells might be a driving force for hepatocellular carcinoma initiation, SOCS3-\(\text{U}^{\text{fl;rox;fert-GFP/wt}}\),\(\text{CD4-Cre}\text{tg/wt}\) mice were crossed to CD4-Cre\(^{\text{tg/wt}}\) [Lee et al., 2001] mice to express GFP in a SOCS3 dependent manner in all T-cell receptor \(\alpha/\beta\) (TCR\(\alpha/\beta\)) expressing T-cells. Acute DEN treatment for 1, 2 and 3 days leads to an increasing GFP fluorescence intensity in isolated, non-parenchymal liver cells (NPLCs) (Fig. 3.12). This demonstrates that SOCS3-\(\text{U}^{\text{fl;rox;fert-GFP/wt}}\),\(\text{CD4-Cre}\text{tg/wt}\) mice can be utilized to identify lymphocyte subpopulations which are specifically activated by IL-6 upon acute DEN treatment.

SOCS3-\(\text{U}^{\text{fl;rox;fert-GFP/wt}}\),\(\text{CD4-Cre}\text{tg/wt}\) mice were subjected to the acute DEN model and their non-parenchymal liver cells were isolated 60h after injection, to elucidate which cell subpopulation exhibits the strong LIGHT mRNA upregulation observed in the liver (Fig. 3.11). \(\frac{7}{8}\) of the isolated non-parenchymal cells were labeled with \(\alpha\)-CD90.2-PE and \(\alpha\)-NK1.1-PE/Cy5 antibodies for FACS sorting (Fig. 3.13), and the remaining \(\frac{1}{8}\) was used for quantitative flow cytometry via
Figure 3.13: **Activation of NK-T cells in the liver upon 60h DEN treatment**

Representative FACS blots of non-parenchymal liver cells isolated from SOCS3-UFlox;frt-GFP/wt, CD4-Cre tg/wt mice, labeled with α-CD90.2-PE and α-NK1.1-PE/Cy5 antibodies. (a) Cells were gated in R1 for lymphocytes and in R2 for T-cells. Cells isolated 60h after treatment with DEN were gated in R3 for NK1.1\textsuperscript{high};GFP\textsuperscript{low} and R4 for NK1.1\textsuperscript{high};GFP\textsuperscript{high} cells. (b) Cells isolated from untreated controls were gated in R3 for NK1.1\textsuperscript{high};GFP\textsuperscript{low} and in R4 for NK1.1\textsuperscript{low};GFP\textsuperscript{low} cells. Quantification of cell numbers for NK1.1\textsuperscript{low};GFP\textsuperscript{low}, NK1.1\textsuperscript{high};GFP\textsuperscript{low} and NK1.1\textsuperscript{high};GFP\textsuperscript{high} cells in (c) absolute cell numbers sorted or as (d) relative cell counts of all cells in R2 in percent. Displayed are means ± SEM, n=5.

MACSQuant (cf. 3.6.4).

Labeled, non-parenchymal liver cells from DEN treated mice were gated for CD90.2\textsuperscript{high} T-cells and sorted into NK1.1\textsuperscript{high};GFP\textsuperscript{low} and NK1.1\textsuperscript{high};GFP\textsuperscript{high} cells (Fig. 3.13a). Isolated T-cells from untreated control animals showed almost no NK1.1\textsuperscript{high};GFP\textsuperscript{high} cells and were therefore sorted into NK1.1\textsuperscript{high};GFP\textsuperscript{low} and NK1.1\textsuperscript{low};GFP\textsuperscript{low} cells (Fig. 3.13b). Quantification of sorted cells reveals around 45% of all liver T-cells in DEN treated mice to be NK-T cells, with 65% of
them being GFP$^{\text{low}}$ and 35% being GFP$^{\text{high}}$ NK-T cells (Fig. 3.13d). On average, 1x10$^4$ NK1.1$^{\text{low}}$;GFP$^{\text{low}}$ T-cells, 5x10$^3$ NK1.1$^{\text{high}}$;GFP$^{\text{low}}$ NK-T cells and 2.5x10$^3$ NK1.1$^{\text{high}}$;GFP$^{\text{high}}$ NK-T cells were sorted and their RNA isolated for gene expression analysis.

### 3.6.3 IL-6 signaling in NK-T cells blocks LIGHT mRNA expression

Gene expression analysis via quantitative real-time PCR of NK1.1$^{\text{low}}$;GFP$^{\text{low}}$ cells, NK1.1$^{\text{high}}$;GFP$^{\text{low}}$ cells and NK1.1$^{\text{high}}$;GFP$^{\text{high}}$ cells reveals a highly significant downregulation of T-cell markers CD8 and CD4 in both NK1.1$^{\text{high}}$;GFP$^{\text{low}}$ and NK1.1$^{\text{high}}$;GFP$^{\text{high}}$ cells compared to NK1.1$^{\text{low}}$;GFP$^{\text{low}}$ cells, confirming that NK1.1$^{\text{low}}$;GFP$^{\text{low}}$ cells are T-cells and the two NK1.1$^{\text{high}}$ populations are NK-T cells (Fig. 3.14). Furthermore, gene expression analysis of IL-6R$\alpha$ demonstrates that, although only a very small subpopulation of NK-T cells express IL-6R$\alpha$ in the basal state (cf. 3.6.1), NK1.1$^{\text{high}}$;GFP$^{\text{high}}$, comprising around 35% of all NK-T cells (Fig. 3.13d), have IL-6R$\alpha$ significantly upregulated compared to NK1.1$^{\text{high}}$;GFP$^{\text{low}}$ NK-T cells that show nearly undetectable levels of IL-6R$\alpha$ expression. In contrast, NK1.1$^{\text{high}}$;GFP$^{\text{low}}$ NK-T cells exhibit a significant 2.5-fold LIGHT upregulation compared to NK1.1$^{\text{low}}$;GFP$^{\text{low}}$ T-cells, whereas NK1.1$^{\text{high}}$;GFP$^{\text{high}}$ NK-T cells express only marginal LIGHT mRNA levels (Fig. 3.14).

Taken together, acute DEN treatment leads to a significant IL-6R$\alpha$ upregulation in about 35% of NK-T cells after 60h, while LIGHT mRNA expression is inhibited in this NK-T cell subpopulation.

### 3.6.4 NK-T cells upregulate IL-6R$\alpha$ upon acute DEN treatment

In order to verify the IL-6R$\alpha$ upregulation in NK-T cells upon acute DEN treatment, isolated, non-parenchymal liver cells from untreated and acute DEN treated SOCS3-U$^{\text{fl}}$;rox$^{\text{frt}}$;GFP$^{\text{wt}}$;CD4-Cre$^{\text{tg}}$;wt mice were labeled with $\alpha$-CD3-VioBlue, $\alpha$-IL-6R$\alpha$-PE and $\alpha$-NK1.1-APC antibodies; dead cells were stained with the Aqua dead cell stain kit and excluded.
Figure 3.14: **LIGHT is mainly expressed by NK-T cells lacking IL-6Rα**

Quantitative real-time PCR analysis of sorted, non-parenchymal liver cells isolated from SOCS3-U^{fl;rox;frt}-GFP/wt;CD4-Cre^{tg/wt} mice 60h after acute DEN treatment. Gene expression is displayed as fold change compared to NK1.1^{low}\;GFP^{low} as means ± SEM, n=5. *** p ≤ 0.001, ** p ≤ 0.01, * p ≤ 0.05

NPLCs were gated for CD3^{high};Aqua^{low} alive T-cells (Fig. 3.15a,b), and no significant differences were observed between untreated and DEN treated animals. T-cells were subsequently analyzed for NK1.1 and IL-6Rα expression (Fig. 3.15c,d) as well as SOCS3-U-dependent GFP expression (Fig. 3.15e). Whereas 50% of hepatic T-cells in untreated mice are NK1.1^{+};IL-6Rα^{-} NK-T cells, acute DEN treatment induces IL-6Rα expression in around 35% of NK-T cells (Fig. 3.15f), consistent with appearance of GFP expression in these T-cells. Taken together, acute DEN treatment induces a significant shift of the hepatic NK-T cell population towards IL-6Rα expression and downstream signaling.

In a reverse assay, IL-6Rα^{fl/fl}, IL-6Rα^{T-KO} and IL-6Rα^{Δ/Δ} mice were subjected to the acute DEN model and NPLCs were isolated after 10 days. Isolated NPLCs were labeled with α-CD3-TexasRed, α-NK1.1-FITC, α-IL-6Rα-PE and α-TCRβ-PE/Cy7 antibodies; dead cells were stained with the Aqua dead cell stain kit.

NPLCs were gated for alive, single-cell T-lymphocytes and analyzed for T-cells and NK-T cells (Fig. 3.16a-c). IL-6Rα^{fl/fl}, IL-6Rα^{T-KO} and IL-6Rα^{Δ/Δ} mice show equal numbers of T-cells and NK-T cells, demonstrating that acute DEN treatment does not affect the distribution of T-cell and NK-T cell populations in an
IL-6Ra-dependent manner. IL-6Ra expression analyses of gated NK-T cells however confirms that a NK-T cell subpopulation from IL-6Ra<sup>fl/fl</sup> mice expresses IL-6Ra, which is not present in both in the whole body or T-cell specific IL-6Ra knock-outs (Fig. 3.16d).

Collectively, while direct, <i>i.p.</i> injection of IL-6 induces acute, hepatic LIGHT mRNA expression, acute DEN treatment first leads to downregulation of hepatic LIGHT mRNA expression for 48 hours after injection, followed by a strong LIGHT upregulation. Concomitantly, acute DEN treatment stimulates IL-6Ra expression in an NK-T cell subpopulation. Interestingly, LIGHT expression is specifically upregulated in the remaining IL-6Ra<sup>−</sup> subpopulation, but downregulated in IL-6Ra<sup>+</sup> cells. However, the exact connection between LIGHT inhibition and IL-6Ra upreg-
Figure 3.16: Lack of NK1.1⁺;IL-6Rα⁺ NK-T cells in IL-6Rα deficient mice
Representative FACS blots of non-parenchymal liver cells isolated from IL-6Rα⁰⁄₀, IL-6Rα^{T-KO} and IL-6Rα^{Δ/Δ} mice, labeled with α-CD3-TexasRed, α-NK1.1-FITC, α-IL-6Rα-PE and α-TCRβ-PE/Cy7 antibodies. Contour blots show T-cells in Q1 and NK-T cells in Q2 from (a) IL-6Rα⁰⁄₀, (b) IL-6Rα^{T-KO} and (c) IL-6Rα^{Δ/Δ} mice. (d) IL-6Rα expression from Q2 NK-T cells is displayed as an histogram overlay analyses, with the IL-6Rα positive subpopulation marked by an arrowhead.

ulation still remains to be elucidated.

Moreover, our experiments also demonstrated that the protective effect of IL-6Rα deficiencies on DEN-induced HCC can be pinpointed to NK-T cells. Only 35% of those cells react to IL-6, as evidenced by IL-6Rα expression and subsequent SOCS3-U-GFP expression. This specific NK-T cell subpopulation is of critical importance for HCC development, and understanding the molecular mechanisms how these cells promote HCC progression might lead to novel therapeutic approaches. Whether LIGHT is the effector molecule of this population remains elusive.

3.7 Dre-mediated recombination in hepatocytes using AlbDre mice

The SOCS3-U construct allows for the Dre-mediated excision of the SOCS3 CDS from the activated SOCS3-U^{Δrox/frt-GFP} allele, in order to generate tissue specific SOCS3 knock-outs. In order to analyze the function of SOCS3 in the liver, a hepatocyte specific Dre driver mouse line under control of the murine albumin promoter, termed AlbDre, was generated. The albumin promoter has already been utilized in generating the hepatocyte specific Cre driver line AlbCre [Postic et al., 1999].
To generate transgenic AlbDre mice via BAC recombineering, a strategy was developed to integrate the Dre CDS and kanamycin resistance cassette into the BAC RP23-301A23, containing the albumin ORF. To this end, the pBAD vector, carrying the viral Redα and Redβ genes, was transformed into RP23-301A23 containing E. coli. Subsequently, Dre CDS and kanamycin resistance cassette were amplified by PCR from the pTE-Dre-neo/kana plasmid [provided by Tim Klöckener, University of Cologne], flanked by 50 bp 5' and 3' homologous regions to exon 2 of the Alb gene, and the PCR product transformed into the E. coli carrying the albumin BAC as well as pBAD plasmid. Successful homologous recombination with the albumin BAC integrates the Dre CDS into exon 2 of the albumin BAC (Fig. 3.17a). DNA from E. coli resistant to chloramphenicol (conveyed by the albumin BAC) and kanamycin (conveyed by the kanamycin resistance cassette introduced by successful recombineering) was isolated and subjected to PCR analysis to identify the wt BAC by an 850 bp band or AlbDre BAC by a 1050 bp band (Fig. 3.17b). Correct AlbDre BAC was purified, sequenced, linearized with PI-SceI and injected into the male pronucleus of a fertilized oocyte to produce AlbDre founder animals. AlbDre founder mice were crossed to SOCS3-U²fl/ro|frt-GFP/wt mice and their recombination efficiency was determined by PCR and Southern-blot analyses.
3.7.1 AlbDre recombines rox-sites specifically in hepatocytes

In order to address the functionality and tissue specificity of the albumin driven Dre line, its ability to recombine the SOCS3-U allele was assessed. To this end, SOCS3-\(U^{fl;rox;frt-GFP/wt}\) mice were crossed to AlbDre\(^{tg/wt}\) mice, with SOCS3-\(U^{fl;rox;frt-GFP/wt}\) as controls. 12w old animals were sacrificed and genomic DNA was extracted from numerous tissues to analyze Dre-mediated DNA recombination by PCR in the designated target tissue as well as off-target tissues (Fig. 3.18a). Further genomic DNA was digested with EcoRV and subjected to Southern-blot analysis. The rox-flanked SOCS3 coding sequence creates a 6.1 kb fragment, also harboring the neomycin resistance cassette and the IRES-GFP (Fig. 3.2c). Dre-mediated recombination excises SOCS3 CDS and decreases the detectable DNA fragment size to 5 kb. DNA fragments were labeled with a DNA probe specific for the neomycin resistance cassette, which also detects the neomycin/kanamycin resistance cassette in the AlbDre construct, where EcoRV digest creates a 5.3 kb fragment (Fig. 3.17a). Dre-mediated recombination of SOCS3-U was detected in hepatocytes with very high efficiency, whereas no recombination occurred in other tissues such as brain, white adipose tissue and skeletal muscle or in hepatocytes without Dre expression (Fig. 3.18b).

This confirms not only that the Dre recombinase is functional and exclusively active in hepatocytes, but also that the SOCS3-U allele can be used with AlbDre to produce a hepatocyte-specific SOCS3 knock-out mice.

3.7.2 Generating homozygous SOCS3\(^L\)-KO mice using AlbDre

To produce hepatocyte-specific, homozygous SOCS3 knock-out (SOCS3\(^L\)-KO) mice, SOCS3-\(U^{Δ;rox;frt-GFP/wt}\) mice were crossed to SOCS3-\(U^{Δ;rox;frt-GFP/wt};AlbDre^{tg/wt}\) mice. To assess Dre-recombination efficiency, primary hepatocytes were isolated from SOCS3-\(U^{Δ;rox;frt-GFP/Δ;rox;frt-GFP}\) and SOCS3-\(U^{Δ;rox;frt-GFP/Δ;rox;frt-GFP};AlbDre^{tg/wt}\) mice and 2\(\times\)10\(^5\) were stimulated in vitro with 50 ng/ml IL-6 for 0, 15, 30, 60 or 120 min.
Results

Figure 3.18: **AlbDre is exclusively active in hepatocytes**

(a) PCR analysis of isolated DNA from hepatocytes (H), brain (B), white adipose tissue (W) and muscle (M) of either SOCS3-U^fl;rox;frt-GFP/wt;AlbDre^tg/wt or SOCS3-U^fl;rox;frt-GFP/wt mice. Dre-mediated recombination decreases the fragment size from 300 bp to 230 bp. (b) Southern-blot analysis of isolated DNA to verify PCR genotyping. Isolated DNA was digested with EcoRV and hybridized with the neo^R probe. EcoRV digest of the SOCS3-U allele gives rise to a 6.1 kb fragment (Fig. 3.2c). Dre-mediated recombination decreases the fragment size to 5 kb. The 5 kb fragment only appears in hepatocytes of SOCS3-U^fl;rox;frt-GFP/wt / AlbDre^tg/wt mice, neither in off-target tissues nor in hepatocytes in the absence of AlbDre. The band between 6.1 kb and 5 kb is derived from the AlbDre neomycin/kanamycin cassette, which also hybridizes with the neomycin probe.

IL-6 stimulation leads to phosphorylation of STAT3 after 15 min both in hepatocytes from SOCS3-U^Δ;rox;frt-GFP/Δ;rox;frt-GFP as well as from SOCS3-U^Δ;rox;frt-GFP/Δ;rox;frt-GFP;AlbDre^tg/wt mice (Fig. 3.19a). SOCS3-U^Δ;rox;frt-GFP/Δ;rox;frt-GFP;AlbDre^tg/wt hepatocytes display even higher pSTAT3 levels throughout the course of stimulation, indicating the absence of negative feedback inhibition by SOCS3 in these hepatocytes. Furthermore, though progression of pSTAT3 and SOCS3 levels decrease over time in SOCS3-U^Δ;rox;frt-GFP/Δ;rox;frt-GFP hepatocytes, phosphorylation of STAT3 in SOCS3-U^Δ;rox;frt-GFP/Δ;rox;frt-GFP;AlbDre^tg/wt hepatocytes is persistently elevated even after 120 min. Consistently, SOCS3 protein levels are reduced in SOCS3-U^Δ;rox;frt-GFP/Δ;rox;frt-GFP;AlbDre^tg/wt hepatocytes at all timepoints investigated. Moreover, analysis of SOCS3 mRNA expression 0, 15 and 120 min after IL-6 stimulation from isolated wt as well as SOCS3-U^Δ;rox;frt-GFP/Δ;rox;frt-GFP;AlbDre^tg/wt primary hepatocyte RNA demonstrates a similar trend, but has to be repeated for several timepoints (Fig. 3.19b).

Taken together, recombination of rox-flanked SOCS3-U by AlbDre is occurring at a high efficiency, as evident by the strong decrease of SOCS3 protein levels upon
Figure 3.19: Prolonged pSTAT3 activity in SOCS3\(^{L-KO}\) mice
(a) Western-blot analyses of primary hepatocytes isolated from
SOCS3-\(U^{\Delta;rox;frt-GFP/\Delta;rox;frt-GFP}\) or SOCS3-\(U^{\Delta;rox;frt-GFP/\Delta;rox;frt-GFP};AlbDre\(^{tg/wt}\) mice using pSTAT3, Tubulin and SOCS3 antibodies. Primary hepatocytes were stimulated with 50 ng/ml IL-6 for 0, 15, 30, 60 or 120 min. (b) RNA was isolated from \(wt\) or SOCS3-\(U^{\Delta;rox;frt-GFP/\Delta;rox;frt-GFP};AlbDre\(^{tg/wt}\) primary hepatocytes stimulated with 50 ng/ml IL-6 for 0, 15 or 120 min and SOCS3 mRNA expression of was analyzed by qPCR. Displayed are means ± SEM, \(n=3\).

IL-6 stimulation in SOCS3-\(U^{\Delta;rox;frt-GFP/\Delta;rox;frt-GFP};AlbDre\(^{tg/wt}\) hepatocytes. Consequently, STAT3 phosphorylation upon IL-6 stimulation is increased and prolonged in the absence of SOCS3 in hepatocytes. In conclusion, combination of AlbDre and SOCS3-\(U^{\Delta;rox;frt-GFP/\Delta;rox;frt-GFP}\) is suited to generate SOCS3\(^{L-KO}\) mice with a tissue-specific, functional SOCS3 knock-out exclusively in hepatocytes.
4 Discussion

Since the 1950’s, dramatic changes in the global population structure led to the aggravation of existing and the emergence of novel health burdens worldwide, a continuous trend that has great impact on health both in industrialized as well as developing regions. The average life expectancy has increased from about 50 years in 1950 to around 70 today and will approach 80 in 2050 [UN Dept. of Economic and Social Affairs 2002]. This development of an ageing population goes hand in hand with a global drop in fertility rate from 5 in 1950 to 2 in 2050. Consequently, a larger portion of the global population will be of advanced age, namely 20% will be above 60 years of age in 2050. A second trend is the ever increasing number of overweight and even obese individuals [Wang et al. 2008], which is ironically the reason why the increase in life expectancy is expected to plateau in around 20 years. Since both ageing and obesity are known risk factors for cancer development, it is tempting to speculate that future populations will on average be older, heavier and suffer from a higher incidence of cancer. The underlying cellular and molecular mechanisms for ageing, obesity and cancer are numerous and so far only partially understood. Basic research uses animal models to investigate connections between different diseases, and increased inflammation lies at the heart of many pathologies, including obesity, cancer and even ageing. The novel inflammatory reporter mouse line SOCS3-U presented in this thesis can help unraveling hitherto unknown mechanisms in inflammation associated diseases. Moreover, SOCS3-U allows for the generation of conditional SOCS3 knock-outs by Dre-mediated recombination.
4.1 The SOCS3-U allele as a novel reporter tool

Cancer research in the last decades has profoundly deepened our understanding of tumor formation and shifted the paradigm of prerequisites towards a broader selection of hallmarks [Hanahan & Weinberg, 2011]. The pro-tumorigenic effect of the tumor microenvironment, encompassing endothelial, epithelial and mesenchymal cells in the near vicinity of cancer cells, as well as immune cells infiltrating the tumor site and the cytokines secreted by these immune cells, have more and more been focussed by recent research efforts.

Inflammatory signaling is largely carried out by secretion of cytokines, a broad category of proteins including interferons, interleukins, colony stimulating factors and chemokines. Immune cells release these factors into the developing tumor to induce apoptosis in cancerous cells and drive proliferation and differentiation in neighboring cells to compensate for the increased cell death. This continuous inflammation however will eventually have detrimental effects on the tumor sites, since deleterious byproducts like reactive oxygen species accumulate and/or because the increased proliferation leads to transformations of hitherto healthy cells [Bisgaard & Thorgeirsson 1996; Maeda & Akaike 1998]. Misguided actions of the immune system have therefore been recognized as a key player in cancerogenesis.

Although it is now known that inflammatory signaling by immune cells contributes to cancer formation, the various cancer entities differ immensely in respect to the exact inflammatory mediator, the source of this mediator and the target cell of the mediator. Thus, our understanding of the signaling machinery responsible for cancer formation in general is still very limited. This thesis therefore aimed at developing a reporter mouse line, which, through conditional gene targeting, should be universally applicable in a large variety of cancer models. The resulting SOCS3-U reporter mouse can visualize inflammatory signaling both in vivo and in vitro with high specificity.
4.1.1 Successful generation of SOCS3-U mice

The SOCS3-U reporter allele couples expression of either an IRES-driven eGFP or the firefly luciferase to expression of SOCS3, a negative feedback regulator of the JAK/STAT pathway in inflammatory signaling. SOCS3-U is targeted to the endogenous SOCS3 locus, to ensure that all regulatory elements of the SOCS3 promoter are applied to expression of SOCS3-U. Correct genomic targeting of the SOCS3-U allele was addressed by Southern-blot analyses, and could be confirmed for two ES-cell clones.

Genetically, SOCS3-U is designed as a knock-out first allele, and activity of SOCS3-U is dependent on Cre-mediated recombination. Upon activation, SOCS3 and an IRES-driven eGFP will be expressed from the SOCS3-U allele under the control of the endogenous SOCS3 promoter. Cre-mediated activation of SOCS3-U and resulting GFP activity after cytokine-stimulation of targeted ES-cells was analyzed with transfection of a Cre-expressing plasmid in vitro prior to injection of ES-cells into blastocysts. SOCS3-U ES-cells demonstrated reproducible but an overall weak GFP fluorescence upon cytokine stimulation. ES-cells in culture are kept pluripotent by addition of leukemia inhibitory factor (LIF), an IL-6-type cytokine inhibiting cell differentiation [Suman et al., 2013]. LIF signals through a heterodimer of one LIF-Receptor β-chain and the common gp130 β-chain, activating the JAK/STAT pathway. The constant exposure of SOCS3-U ES-cells to LIF signaling results in high, basal levels of SOCS3 [Naka et al., 1997], thereby compromising the SOCS3-GFP induction by cytokine stimulation and resulting in the rather low shift in GFP fluorescence.

4.1.2 SOCS-U^{fl;rox;frt-GFP/wt} mice show wt inflammation response

The precondition of Cre-mediated recombination to activate SOCS3-U and concomitantly expression of SOCS3 from the SOCS3-U allele necessitates that the off-target tissues are functionally SOCS3 haploinsufficient. In order to ensure that presence of the non activated SOCS-U^{fl;rox;frt-GFP} allele does not cause sig-
nificant aberrations in the inflammation response, primary hepatocytes from SOCS-U^{Rox/frt-GFP/wt} mice were isolated and their response to IL-6 stimulation was compared to wild-type hepatocytes. IL-6 stimulation led to comparable phosphorylation of STAT3 and the resulting SOCS3 upregulation showed an equal capability to inhibit further pSTAT3 activity after induction of SOCS3. Thus, one SOCS3 allele can inhibit the JAK/STAT pathway as efficient as two alleles.

4.1.3 SOCS3-U visualizes inflammation in a Cre-dependent manner

Taken together, the novel SOCS3-U reporter mouse line allows for the visualization of numerous inflammatory processes \textit{in vitro} and \textit{in vivo}. SOCS3, and concomitantly also the eGFP or luciferase reporter respectively, is upregulated not only by IL-6 type cytokines or TNF\(\alpha\) via NF-\(\kappa\)B as classical inflammatory mediators, but also by leptin and insulin, opening up versatile possibilities to monitor metabolic processes. It is however noteworthy that whole-body SOCS3 haploinsufficiency conveys a certain level of protection from diet-induced obesity via an increased leptin sensitivity [Howard \textit{et al.}, 2004].

Since the activity of SOCS3-U is conditionally controlled by Cre-mediated recombination, utilization of SOCS3-U can take full advantage of the wide array of available Cre-driver lines. Accordingly, SOCS3-U can be activated specifically in defined immune cell populations, neuronal cells or parenchymal tissues. Additionally, AAV-delivered Cre-recombinase or inducible Cre driver can activate SOCS3-U even in adult animals in a defined manner.

4.2 M2 TAMs attract T-cells to the colon upon AOM/DSS treatment

Colorectal cancer constitutes a major health burden worldwide, especially in industrialized countries, and predominantly develops as a consequence of chronic inflammation in the bowel. Chronic inflammation can be caused by chronic in-
fections, tobacco smoking or other pollutants, or dietary factors such as obesity. Patients already suffering from inflammatory bowel diseases, such as Crohn’s disease or ulcerative colitis, have a strongly increased risk of developing colorectal cancer. Progression of colorectal cancer as a classic case of colitis associated cancerogenesis is recapitulated in the AOM/DSS mouse model [Tanaka et al., 2003]. Injection of the pro-carcinogen AOM is coupled to supplementation of the drinking water with DSS, which is a strong inducer of colitis.

The cancer promoting effect of the AOM/DSS model can be abrogated in genetic mouse models, in which inflammatory signaling is disturbed. Deletion of IKKβ in cells of the myeloid lineage reduces the inflammatory tone and alleviates the tumor burden after AOM/DSS treatment [Greten et al., 2004]. Experiments from our own lab demonstrate that abrogation of IL-6 signaling in IL-6Rα deficient mice (IL-6RαΔ/Δ) reduces colorectal tumor formation in the AOM/DSS model [Claudia Wunderlich, University of Cologne]. Furthermore, transcriptome analysis of IL-6RαΔ/Δ tumors revealed significant differences compared to IL-6Rα proficient tumors, such as an overall reduction in lymphocyte markers as well as decreased CCL20 and CCR6 expression. Additional data showed that loss of IL-6Rα on myeloid cells abrogates alternative macrophage activation towards the M2 subtype [Mauer et al., 2014]. Collectively, these observations prompted the hypothesis that IL-6 signaling driven M2 macrophages, upon increased inflammation in the colon, express CCL20, which attract CCR6 expressing lymphocytes [Baba et al., 1997; Hieshima et al., 1997], promoting colorectal cancer development.

Utilization of SOCS3-Ufl;rox;fRT-GFP/wt;R26-fl-tdTomatofl/wt;LysM-Crewg/wt mice in the AOM/DSS model enabled the identification of M1 and M2 macrophages in order to elucidate the contribution of the respective subpopulations to colorectal cancer formation. Expression of tdTomato in LysM-Cre expressing myeloid cells resulted in red fluorescence both in M1 and M2 macrophages, whereas expression of eGFP from the SOCS3-U allele in LysM-Cre expressing myeloid cells however was dependent on stimulation of SOCS3. Hence, fluorescence activated cell sorting allowed
for the separation of tdTomato\textsuperscript{high}\textsuperscript{;}GFP\textsuperscript{low} M2-like and tdTomato\textsuperscript{high}\textsuperscript{;}GFP\textsuperscript{high} M1-like macrophages for in depth analysis.

SOCS3 is highly expressed in infiltrating macrophages \cite{Liu2008} and shows a strong correlation with M1 macrophages \cite{Arnold2014}. Data from this study now could demonstrate the absence of IL-6R\alpha expression in tdTomato\textsuperscript{high}\textsuperscript{;}GFP\textsuperscript{low} M2-like macrophages and a concomitant, unaltered IL-6R\alpha expression in tdTomato\textsuperscript{high}\textsuperscript{;}GFP\textsuperscript{high} M1-like macrophages, corroborating a polarized IL-6R\alpha expression in M2 macrophages and lack of IL-6R\alpha expression in M1 macrophages. Interestingly, CCL20 mRNA expression exhibited a similar pattern towards M2 macrophages, substantiating our initial hypothesis of M2 macrophages predominantly expressing CCL20 upon AOM/DSS treatment.

In addition to the converse CCL20 and IL-6R\alpha expression in M1- and M2-like macrophages, tdTomato\textsuperscript{high}\textsuperscript{;}GFP\textsuperscript{low} M2-like macrophages exhibited a strikingly elevated IL-17RA expression. Our initial hypothesis was that IL-6 drives CCL20 expression in macrophages. However, stimulation of BMDMs and qPCR of CCL20 led to undetectable CCL20 levels upon stimulation, thereby excluding IL-6 as an activator of CCL20 expression. Thus, we proposed that macrophage polarization towards M2 as already described leads to the susceptibility to express CCL20, by upregulation of IL-17RA. IL-17RA is one of five IL-17 receptor subunits and forms heterodimers with IL-17RC, transducing signaling evoked by IL-17A and IL-17F, two of the six IL-17 cytokines, ligand binding \cite{Gaffen2009}. IL-17A and IL-17F play a crucial role in recruitment and activation of immune cells, inducing the expression of pro-inflammatory cytokines and chemokines, including CCL20 \cite{Iwakura2011}.

Thus, our current hypothesis is that IL-17 produced by T-cells activates CCL20 expression in IL-17RA expressing M2 macrophages and thereby creates a vicious cycle of inflammation that drives CRC development. Additionally, IL-17 can signal directly on intestinal epithelial cells, promoting cell growth and survival. It is intriguing to speculate that T\textsubscript{H}17 and \gamma\delta T-cells release IL-17 cytokines with differ-
ent target cells, both acting in concert and driving colorectal cancer development. Further investigation of this hypothesis will lead to novel therapeutic strategies to combat the continuing spread of colorectal cancer in the coming years.

4.3 Activation of the tumor microenvironment in DEN induced HCC

Hepatocellular carcinoma account for 85-90% of primary liver cancers, representing a large global health burden, particularly in males of less developed countries [El-Serag, 2011]. HCC usually develops gradually from chronic hepatitis, through either dietary or alcoholically induced, steatotic liver into liver fibrosis, to liver cirrhosis and ultimately to HCC. Common to all the initial causes for HCC development is increased inflammation, marking HCC as an inflammation associated cancer. Persistent liver inflammation causes hepatocyte death, inducing a compensating hyperproliferation of neighboring hepatocytes, a driving force for cancerogenesis [Bisgaard & Thorgeirsson, 1996]. HCC development can be recapitulated in a mouse model of DEN injection into 12-15 days old, male mice, which develop tumors after 8 months [Vesselinovitch & Mihailovich, 1983]. Furthermore, 8 week old male mice can be injected with an acute dose of DEN, mimicking the increased inflammation occurring in the tumor initiation phase.

Abrogation of inflammatory signaling protects against DEN-induced HCC

Numerous observations implicate immune cell action and inflammatory signaling in HCC development. Abrogation of IL-6 signaling was demonstrated to protect from DEN-induced HCC formation, either in whole-body IL-6 knock-out [Park et al., 2010] or by IL-6Ra deficiency (IL-6RaΔ/Δ) [Gruber et al., 2013]. Kupffer cell derived IL-6 after hepatocyte death has been shown to aggravate HCC development, indicating a contribution of Kupffer cells to liver tumor formation [Naugler et al., 2007]. Interestingly, contribution of IL-6 signaling to HCC formation does not
occur directly on hepatocytes, as IL-6Rα deficiency specifically in hepatocytes does not protect form DEN-induced HCC development; however, the protective effect of whole-body IL-6Rα deficiency is recapitulated in a T-cell specific IL-6Rα ablation model [Gruber, 2013]. Despite these observations of the influence of Kupffer cells as well as lymphocytes on hepatocellular carcinoma development, the exact contribution and interplay between the respective immune cell populations remains to be elucidated.

4.3.1 Unraveling macrophage subpopulations in DEN induced HCC using SOCS3-U

Tumor associated macrophages are important mediators of cancer-related inflammation and comprise a large part of infiltrating immune cells [Solinas et al., 2009]. Consistently, signaling by macrophages has been implicated on numerous occasions with HCC development [Capece et al., 2013]. IL-6 represents a critical inflammatory mediator of TAM signaling, in that TAM derived IL-6 promotes TH17 expansion, which suppresses cytotoxic T-cell activity [Kuang et al., 2010; Zhao et al., 2011]. In addition to M1 and M2 macrophages, inflammatory signaling by liver resident Kupffer cells affects HCC formation. Interestingly, IL-6 signaling neither in infiltrating M1 nor in M2 macrophages contributes to DEN-induced HCC formation, as demonstrated by unaltered tumor formation in IL-6Rαfl/fl;LysM-CreαKg/wt mice, but might impact Kupffer cells, as they are not affected by LysM-Cre mediated recombination [Hume, 2011].

Although Kupffer cells are not affected by LysM-Cre expression, they still express classic macrophage markers such as F4/80 [Austyn & Gordon, 1981]. Isolation of non-parenchymal liver cells from SOCS3-Ufl;rox;frt-GFP/wt;R26-fl-tdTomatofl/wt;LysM-CreαKg/wt mice subjected to the chronic DEN model and subsequent labeling with an F4/80 antibody therefore allowed for the identification of tdTomato\textsuperscript{high};GFP\textsuperscript{high} M1 macrophages, tdTomato\textsuperscript{high};GFP\textsuperscript{low} M2 macrophages and tdTomato\textsuperscript{low};F4/80\textsuperscript{high} Kupffer cells.
Parallel transcriptome analysis of all three macrophage subpopulation after chronic DEN treatment should give novel insight into the crosstalk between myeloid cells, epithelial cells and lymphocytes in hepatocellular carcinomas. This task is still under investigation.

4.3.2 Acute DEN treatment induces IL-6Rα proficient NK-T cells

Previous data from our lab already demonstrated a central role for lymphocytes in HCC development in an IL-6 dependent manner, in that IL-6Rα deficiency on T-cells protects from DEN-induced HCC development [Gruber, 2013]. Increased liver steatosis upon CD-HFD feeding, mimicking a tumor development promoting environment, increases hepatic T-cell numbers, including NK-T cells as well as T_{reg}s, and drives elevated expression of IL-1, IL-17 and LIGHT [Wolf et al., 2014]. NK-T cell secreted LIGHT has been demonstrated to be pivotal for hepatic lipid uptake in this context, ultimately constituting a driving force for HCC development.

Data from this study revealed additional molecular mechanisms linking active and NK-T cell function to DEN-induced HCC development. mRNA expression analysis of NPLCs isolated from SOCS3-U^{frx;frt-GFP/wt;CD4-Cre^{tg/wt}} mice subjected to the acute DEN model demonstrated that approximately 35% of NK-T cells up-regulate IL-6Rα upon DEN treatment, whereas non activated NK-T cells do not express IL-6Rα. In line with this experiment, analysis of NPLCs isolated from control and IL-6Rα deficient mice after acute DEN treatment substantiated an emergence of an IL-6Rα^+ NK-T cell subpopulation. Unexpectedly, LIGHT mRNA expression was significantly higher in the remaining IL-6Rα^- NK-T cells subpopulation. It is however reasonable to assume that the alteration of LIGHT mRNA expression upon acute DEN treatment follows a similar mechanism, since increased IL-6 levels were previously demonstrated to be delayed after acute DEN injection [Gruber et al., 2013].

Collectively, acute DEN injection separates hepatic NK-T cells into two subpop-
ulations, one IL-6Rα deficient population expressing LIGHT, and one IL-6Rα proficient population with an elusive function that promotes HCC development. It is intriguing to speculate that both populations function in concert with active effector T-cells to drive hepatocellular carcinoma initiation. Increased levels of IL-1 and IL-17 in the CD-HFD model indicate a possible role for T_{H}17 cells to be the respective effector T-cell population, corroborated by a TAM-derived IL-6 mediated T_{H}17 cell increase. Continuing analysis of the hitherto unidentified IL-6Rα expressing NK-T cell subpopulation, the upstream mediator of LIGHT expression by IL-6Rα deficient NK-T cells and additional hepatocellular target genes will give further insights into the intricate network of lymphocyte interaction in HCC development.

4.4 AlbDre is a new tissue-specific Dre-driver line

Genetic mouse models represent a cornerstone of basic research in the 21st century and are a constant source of discoveries with implications for applied research, pharmaceutical sciences and clinical trials. Efficient genetic analyses relies on specific genetic tools, to modify genes and study their impact on development, pathologies or maintenance of a functional organism.

Conditional gene targeting, that is genetic modification using site specific recombinases, enables a wide array of possible gene modifications, including gene disruption, activation or overexpression [Rajewsky et al., 1996]. Site specific recombination requires the modification of the target gene with recombinase target sites, depending on the desired effect after recombination. The two most common applications for site specific recombination are conditional gene knock-outs, where usually several exons are flanked by recombinase target sites and will be excised after recombination, or conditional knock-ins, where expression of the gene of interest is inhibited by a target site flanked transcriptional stop cassette until recombination activates gene expression. The Cre recombinase from bacteriophage P1 and the corresponding loxP recombination target sites have emerged as the primary site specific recombination system in the last two decades, combining high efficiency
with high versatility.

More sophisticated approaches in conditional gene targeting might need a second site specific recombination system with similar requirements concerning versatility and efficiency. A distinct recombination system can e.g. be used to simultaneously modify two different target alleles in one mouse, or to enhance the specificity of a construct by tandem application of both recombination systems. The Dre/rox site specific recombination system from bacteriophage D6 matches these criteria and can be utilized in parallel or in concert with Cre/loxP [Sauer & McDermott, 2004; Anastasiadis et al., 2009]. This study aimed at creating a tissue-specific Dre-driver line, expressing the Dre-recombinase specifically in hepatocytes using the albumin (Alb) promoter.

The Alb promoter was chosen to express Dre-recombinase specifically in hepatocytes, as it has been already successfully applied to express Cre-recombinase [Postic et al., 1999]. Alb encodes for serum albumin, a protein produced exclusively in the liver and secreted into the blood stream. Expression of the albumin promoter starts at embryonic day 9.5 (E9.5), and is increasing with full development of the liver [Kellendonk et al., 2000]. Recombination efficiency in the liver of albumin-driven Cre-recombinase has been found to be 80%, with no recombination in any other tissue analyzed, while a closer analysis of target protein levels exclusively in hepatocytes after Alb-Cre mediated recombination revealed even a reduction by 95% [Postic et al., 1999].

A BAC containing the albumin ORF and the complete promoter including all regulatory elements was used to generate the transgenic mouse line by recombinneering in E. coli [Zhang et al., 1998]. To this end, the Dre-recombinase CDS was amplified by PCR and introduced into the Alb-BAC by homologous recombination, mediated by the recombination system Red\(\alpha\) and Red\(\beta\) transformed into the BAC carrying bacteria. Successful integration of the Dre-recombinase CDS into exon 2 of the Alb ORF was verified by PCR and sequencing and the recombined AlbDre BAC injected into the male pronucleus of fertilized oocytes. Integration of
the recombined BAC occurs randomly and potentially multiple times, so that every positive founder mouse constitutes a separate transgenic mouse line, and recombination efficiency has to be determined both for the target tissue as well as off-target tissues.

The ability of AlbDre to recombine DNA in vivo was addressed in SOCS3-Ufl;rox;frt-GFP/wt;AlbDre$^{tg/ wt}$ mice by Southern-Blot analysis. Genomic DNA was isolated from hepatocytes, brain tissue, white adipose tissue and muscle tissue, and recombined DNA was exclusively detected in hepatocellular DNA. The Southern-Blot analysis revealed a very high recombination efficiency for AlbDre, with at least 95% of the DNA being successfully recombined.

To use the AlbDre mouse to conditionally inactivate SOCS3 in hepatocytes, the SOCS3-Ufl;rox;frt-GFP allele had to be activated, in order to determine the recombination efficiency of AlbDre on the protein level. Hence, hepatocytes from SOCS3-U$^{\Delta,rox;frt-GFP/\Delta,rox;frt-GFP}$ and SOCS3-U$^{\Delta,rox;frt-GFP/\Delta,rox;frt-GFP, AlbDre^{tg/wt}}$ (SOCS3$^{L-KO}$) mice were isolated and stimulated ex vivo with IL-6 to drive SOCS3 expression. SOCS3$^{L-KO}$-hepatocytes exhibited a significantly delayed upregulation of SOCS3 upon IL-6 stimulation, confirming a high recombination efficiency for AlbDre. Consistently, STAT3 phosphorylation was significantly prolonged in SOCS3$^{L-KO}$-hepatocytes. The eventual expression of SOCS3 even in SOCS3$^{L-KO}$-hepatocytes can be attributed to remaining hepatocytes, that have escaped Dre mediated recombination.

Taken together, a hepatocyte specific SOCS3 knock-out can be generated using SOCS3-U$^{\Delta,rox;frt-GFP/\Delta,rox;frt-GFP}$ and AlbDre$^{tg/wt}$ mice, which can be combined with an additional Cre-dependent knock-out of any gene of interest in another cell type.
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6 Appendix

6.1 Plasmid maps

6.1.1 SOCS3-U targeting construct

Figure 6.1: The SOCS3-U targeting construct (22473 bp) with notable features. The left arm of homology (LAH), containing the untranslated exon 1, and the right arm of homology (RAH), containing the 3'UTR, flank the knock-in features. The regulatory region contains a loxP flanked neomycin resistance cassette and the SV40 polyadenylation signal. SOCS3 exon 2, including 5'UTR (in gray) and the SOCS3 coding sequence (black arrow), is flanked by rox sites and followed by the reporter region. The reporter contains an IRES driven GFP, flanked by FRT sites in tandem orientation, and the bovine growth hormone polyadenylation signal. The firefly luciferase coding sequence is encoded on the antisense strand and followed by a FRT site in opposite direction to the other two. Motifs necessary for bacterial replication are depicted together with the AsiSI restriction site for linearization prior to transfection.
6.1.2 SERCA plasmid

Figure 6.2: Stop-eGFP-ROSA-CAGs (SERCA)

Stop-eGFP-ROSA-CAGs (SERCA, 16056 bp) with notable features. AscI restriction site lies in front of the FRT flanked IRES-GFP. NheI and EcoRV restriction sites are used for further cloning. Motifs necessary for bacterial replication are also depicted. [Klisch 2006]
6.1.3 GK12TK plasmid

Figure 6.3: GK12TK plasmid (7314 bp) with notable features. AvrII and PmeI restriction sites are used for further cloning. Motifs necessary for bacterial replication are also depicted. [Wunderlich et al., 2010]
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Köln, Mai 2015

(Philipp Justus Ackermann)
Curriculum vitae

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MASTERARBEIT

Titel | Phenotypic characterization of pcp1∆ mutants and analysis of their genetic interactions in *Saccharomyces cerevisiae*

Betreuer | Prof. Dr. Thomas Langer

BACHELORARBEIT

Titel | Genetic characterization of YLR168c in *Saccharomyces cerevisiae*

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