

Impact of IL-6 signaling on pancreatic and colorectal cancer

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*Zur Erinnerung an Puschel,
die mich gelehrt hat,
dass ein dankbares Herz wichtiger ist als ein gesunder Körper
und dass das Leben ein Geschenk ist, das voller Wunder steckt.*

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List of Abbreviations

ADM	acinar-to-ductal metaplasia
ANOVA	analysis of variance
ATCC	American Type Culture Collection
CAC	colitis-associated cancer
CEA	carcinoembryonic antigen
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule1
CEACAM5	carcinoembryonic antigen-related cell adhesion molecule5
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule6
CEAR	carcinoembryonic antigen receptor
ChIP	chromatin immunoprecipitation
COX-2	cyclooxygenase-2
CRC	colorectal cancer
CTM	chetomin
DAPI	4',6-diamidino-2-phenylindole
DFO	deferoxamine mesylate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DR5	death receptor5
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
EMT	epithelial-mesenchymal transition
FACS	fluorescence-activated cell sorting, commonly used for flow cytometry
FBS	fetal bovine serum
FFPE	formalin-fixed and paraffin-embedded
gp130	glycoprotein 130
HIF-1 α	hypoxia-inducible factor 1 α

List of Abbreviations

HRP	horseradish peroxidase
i.p.	intraperitoneal
IBD	inflammatory bowel disease
IL-6	interleukin-6
IL-6R	IL-6 receptor
KO	knock out
MAPK	mitogen-activated protein kinase
mIL-6R	membrane-bound form of the IL-6R
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	natural killer cells
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate-buffered saline
PDAC	pancreatic ductal adenocarcinoma
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PMA	phorbol-12-myristate-13-acetate
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SCID/bg	severe combined immunodeficient beige
sgp130	soluble gp130
sIL-6R	soluble IL-6 receptor
STAT3	signal transducer and activator of transcription-3
TBS	tris-buffered saline
TGF- β	transforming growth factor beta

Abstract

The present work describes the inhibition of the inflammatory cytokine interleukin-6 (IL-6) as a promising treatment option for pancreatic and colorectal cancer (CRC). Both cancer entities are associated with chronic inflammation, and IL-6 contributes to the tumorigenesis of both. IL-6 signals *via* two different mechanisms: In classic signaling, IL-6 binds to the membrane-bound IL-6 receptor (mIL-6R), which subsequently recruits two molecules of the signal-transducing glycoprotein 130 (gp130). While mIL-6R is only expressed on a few cell types, gp130 is ubiquitously expressed. In trans-signaling, IL-6 interacts with a soluble form of IL-6 (sIL-6R). This complex binds to gp130, and can thus signal on cells that do not express mIL-6R themselves. Trans-signaling is naturally inhibited by a soluble form of gp130 (sgp130).

Pancreatic cancer shows the worst 5-year survival rate of all common types of cancer, and new therapeutics are urgently needed. In the first part of this study, it was examined whether pancreatic tumor growth was decreased by inhibition of IL-6 signaling in orthotopic xenograft models of pancreatic cancer. We used a conservative as well as an adjuvant treatment scheme. In the conservative scheme, the human pancreatic cancer cells Colo357 or PancTuI were inoculated into the pancreas of immunodeficient mice, and treatment started three days later. The tumor cell inoculation was also done in the adjuvant setting, which mirrors the situation of patients with resectable tumors. In this setting, the primary tumor was removed after ten days, and therapy started after resection.

Inhibition of both, classic and trans-signaling, by the anti-IL-6R antibody tocilizumab as well as specific inhibition of trans-signaling by sgp130Fc (a fusion molecule of sgp130 with the constant portion of a human antibody) significantly reduced tumor growth and metastasis in the conservative and adjuvant setting. These findings demonstrate that the inhibition of trans-signaling is sufficient for therapy and that additional blockade of classic signaling, which presumably has more side effects, does not show any further tumor reduction.

The second part of this thesis deals with CRC, the second leading cause of cancer-related deaths in Europe. One of the best-known tumor-associated antigens is carcinoembryonic antigen-related cell adhesion molecule5 (CEACAM5), which is involved in cell adhesion,

migration, tumor invasion and metastasis. Its family member CEACAM6 also contributes to metastasis and is an independent predictor of poor survival in CRC. Some studies indicated a link between IL-6 and CEACAM5, but the literature is controversial and no studies described the role of trans-signaling or the effect of IL-6 on CEACAM6. As CEACAM5 and CEACAM6 are closely related and exhibit similar functions, the impact of IL-6 signaling on the expression of CEACAM5 and CEACAM6 was analyzed.

While classic IL-6 signaling only slightly increased the expression of CEACAM5/6 in the examined colon cell lines, trans-signaling markedly upregulated their expression. This probably occurs due to a low mIL-6R expression on the examined colon cells. The CEACAM5/6 upregulation depended on the phosphorylation of the signal transducer and activator of transcription-3 (STAT3), an early event in IL-6 signaling. It is known that activated STAT3 increases the protein levels of hypoxia-inducible factor 1 α (HIF-1 α), which was confirmed for IL-6 trans-signaling in colon cells. As treatment with the HIF-1 α stabilizer deferoxamine mesylate (DFO) also upregulated CEACAM5/6, we assumed that HIF-1 α plays a role in the IL-6-mediated CEACAM5/6 upregulation. Yet, IL-6 trans-signaling did not increase the expression of the classical HIF-1 α target genes CA9 and VEGF, and experiments with an inhibitor of HIF-1 α transcriptional activity (chetomin) showed contradictory results. Therefore, HIF-1 α might be involved in the connection of IL-6 and CEACAMs, but its distinct function in this context remains unclear and needs to be further examined. As CEACAM5 and CEACAM6 exhibit pro-tumorigenic functions and are upregulated by IL-6 trans-signaling, inhibition of trans-signaling could be a therapeutic strategy for CRC.

In summary, this study emphasizes IL-6 as a promising target for the therapy of pancreatic and colorectal cancer. Mainly trans-signaling seems to be responsible for the pro-tumorigenic effects of IL-6. Specific inhibition of trans-signaling presumably has less side effects than blockade of both pathways, and thus, inhibition of trans-signaling should be further evaluated in pre-clinical *in vivo* models of CRC and in clinical studies for the treatment of pancreatic cancer.

Zusammenfassung

Die vorliegende Arbeit beschreibt die Inhibition des inflammatorischen Zytokins Interleukin-6 (IL-6) als vielversprechende Therapie-Option für das Pankreas- und Kolorektalkarzinom. Beide Krebsarten weisen eine Assoziation mit chronischer Entzündung auf und IL-6 ist an der Krebsentstehung beider Entitäten beteiligt. IL-6 kann über zwei verschiedene Signalwege wirken: Beim klassischen Signaling bindet IL-6 an den membrangebundenen IL-6-Rezeptor (IL-6R), der anschließend zwei Moleküle des signalweiterleitenden Co-Rezeptors Glykoprotein 130 (gp130) rekrutiert. Im Unterschied zum IL-6R, der nur auf wenigen Zellarten vorhanden ist, wird gp130 auf allen Zellen exprimiert. Beim IL-6-Trans-Signaling interagiert IL-6 mit einer löslichen Form des IL-6R (sIL-6R). Dieser Komplex bindet im Anschluss an gp130 und kann dadurch auch auf Zellen wirken, die selbst keinen IL-6R bilden. Trans-Signaling wird durch eine natürlich vorkommende lösliche Form von gp130 (sgp130) inhibiert.

Pankreaskrebs weist unter allen häufig auftretenden Krebsarten die schlechteste 5-Jahres-Überlebensrate auf, weshalb neue Therapieoptionen dringend benötigt werden. Im ersten Teil dieser Dissertation wurde in einem orthotopen Xenograft-Modell untersucht, ob die Hemmung des IL-6 Signalweges das Tumorwachstum von Pankreastumorzellen reduziert. Sowohl ein konservatives als auch ein adjuvantes Therapie-Schema wurden analysiert. Beim konservativen Schema begann die Therapie drei Tage nach der Inokulation der humanen Pankreaskrebszellen Colo357 oder PancTuI in das Pankreas von immundefizienten Mäusen. Die Tumorzellinokulation fand auch im adjuvanten Setting statt, das ein Modell für resektable Tumoren ist, jedoch wurde hier der Primärtumor nach zehn Tagen wieder entfernt und die Therapie begann erst nach der Resektion.

Sowohl die Behandlung mit dem Anti-IL-6R-Antikörper Tocilizumab, der klassisches und IL-6-Trans-Signaling hemmt, als auch eine spezifische Inhibition des Trans-Signaling durch sgp130Fc (ein Fusionsmolekül aus sgp130 und dem Fc-Teil eines humanen Antikörpers), reduzierte das Tumorwachstum und die Metastasierung im konservativen und adjuvanten Therapiemodell signifikant. Diese Ergebnisse zeigen, dass die Inhibition des Trans-Signaling für eine Therapie ausreicht, und eine zusätzliche Hemmung des klassischen Si-

gnalweges keine weitere Tumorreduktion hervorruft.

Der zweite Teil der vorliegenden Dissertation beschäftigt sich mit dem Kolorektalkarzinom, das in Europa die zweithäufigste Todesursache unter den Krebserkrankungen darstellt. Eines der bekanntesten tumorassoziierten Antigene des Kolorektalkarzinoms ist CEACAM5, das an der Zelladhäsion, Migration, Tumorinvasion und Metastasierung beteiligt ist. Das verwandte Molekül CEACAM6 trägt ebenfalls zur Metastasierung bei und ist ein unabhängiger prognostischer Faktor für kürzeres Überleben. Einige Studien konnten bereits einen Zusammenhang zwischen IL-6 und der CEACAM5-Expression aufzeigen, doch die Literatur ist an dieser Stelle kontrovers und bisher wurde weder die Bedeutung des Trans-Signaling noch die Verbindung zwischen IL-6 und CEACAM6 untersucht. Da CEACAM5 und CEACAM6 miteinander verwandt sind, erschien eine Analyse des Einflusses der IL-6-Signalwege auf die Expression von CEACAM5 und CEACAM6 vielversprechend.

Während das klassische IL-6-Signaling die Expression der pro-tumorigenen Moleküle CEACAM5/6 in den untersuchten Kolon-Zelllinien nur geringfügig erhöhte, führte IL-6-Trans-Signaling zu einer stark erhöhten Expression. Dies resultiert vermutlich aus einer geringen Expression des IL-6R auf Kolon-Zellen. Die erhöhte CEACAM5/6-Expression war von der Phosphorylierung des Transkriptionsfaktors STAT3 abhängig, einem frühen Ereignis im IL-6 Signalweg. Es ist bekannt, dass STAT3 das Level von HIF-1 α auf Proteinebene erhöht, was *in vitro* für das IL-6-Trans-Signaling in Kolon-Zellen bestätigt werden konnte. Da eine Behandlung mit dem HIF-1 α -Stabilisator Deferoxamin Mesylat ebenfalls die Expression von CEACAM5/6 steigerte, lag der Schluss nahe, dass HIF-1 α an der IL-6-vermittelten CEACAM5/6 Hochregulierung beteiligt ist. Allerdings führte das IL-6-Trans-Signaling nicht zu einer verstärkten Expression von klassischen HIF-1 α -Zielgenen wie CA9 und VEGF, und Experimente mit einem Inhibitor der transkriptionellen HIF-1 α -Aktivität (Chetomin) zeigten widersprüchliche Ergebnisse. Daher ist HIF-1 α zwar sehr wahrscheinlich an der IL-6-induzierten CEACAM5/6 Überexpression beteiligt, doch die genaue Funktionsweise in diesem Signalweg ist noch unklar.

Zusammenfassend beschreibt diese Dissertation IL-6 als ein vielversprechendes Zielmolekül für die Therapie des Pankreas- und Kolorektalkarzinoms. Vor allem das IL-6-Trans-Signaling scheint für die pro-tumorigenen Effekte verantwortlich zu sein. Eine spezifische Inhibition des Trans-Signaling hat vermutlich weniger Nebenwirkungen als eine Hemmung beider Signalwege und sollte daher in präklinischen *in vivo*-Modellen für das Kolorektalkarzinom und in klinischen Studien für die Therapie des Pankreas-Karzinoms weiter erforscht werden.

Chapter 1

General introduction

1.1 Inflammation as a hallmark of cancer

Cancer is the second leading cause of death in member states of the European Union, accounting for 24% of all deaths in 2011 [1]. Among men, colorectal cancer is the second most common cause of death from cancer (11.6 %) after lung cancer (26.1 %) and followed by prostate cancer (9.5 %), stomach cancer (6.5 %) and pancreatic cancer (5.4 %) [2]. Pancreatic and colorectal cancer are also among the five most frequent causes of cancer-related deaths among women, with colorectal cancer ranking second (13.0 %) after breast cancer (16.8 %) and followed by lung cancer (12.7 %), pancreatic cancer (6.7 %) and stomach cancer (5.6 %) [2]. Overall, the cancer mortality has continuously decreased during the last twenty years [3]. Besides changes in smoking behavior, the key contributors to these falling trends are early diagnosis and screening as well as improved therapies and disease management [3]. Yet, this does not apply for pancreatic cancer [3].

In 2000, Hanahan and Weinberg described in a seminal paper six traits of cancer cells which they called "the hallmarks of cancer". They comprised sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, resisting cell death, inducing angiogenesis, and activating invasion and metastasis [4]. In the following decade, remarkable progress in cancer research revealed that these traits of cancer cells are not sufficient to understand tumor biology. Instead, the cells of the tumor-associated stroma (the tumor microenvironment) became more and more recognized as active participants in tumorigenesis [5]. Hanahan and Weinberg revisited their paper in 2011, adding two "emerging hallmarks of cancer" to their list of traits: deregulating cellular energetics and avoiding immune destruction. Moreover, they described "enabling characteristics", which enable the acquisition of the cancer hallmarks. One of them is genome instability and mutation, and the other one tumor-promoting inflammation.

Already in 1986, Dvorak called tumors "wounds that do not heal" and described similarities between the generation of the tumor stroma and inflammatory processes involved in

wound healing [6]. Meanwhile we know that virtually all neoplasms contain immune cells in their microenvironment [5]. Historically, these immune cells were believed to represent anti-tumor activities of the body, and indeed, many tumors elicit anti-tumoral responses of the immune system [5].

But it became clear that the immune system plays a dual role in cancer: On the one hand, immune cells can suppress tumor growth by killing cancer cells or inhibiting their outgrowth, but on the other hand, they can also promote tumor progression by establishing pro-tumorigenic conditions within the tumor microenvironment [7, 8]. Immune cells and inflammation in general can contribute to the acquisition of multiple cancer hallmarks by supplying bioactive molecules to the tumor microenvironment, including growth, survival and pro-angiogenic factors and extracellular matrix-modifying enzymes [5]. One family of such molecules are the cytokines, proteins of low molecular weight that mediate cell-to-cell communication [9]. Cytokines also play a dual role in tumorigenesis: They can suppress tumor formation by controlling infection, inflammation and immunity, but they can also be exploited by tumor cells to promote growth, increase resistance to apoptosis and foster dissemination [10].

1.2 Inflammation in gastrointestinal tumors

Chronic inflammation is especially involved in the tumorigenesis of gastrointestinal and hepatobiliary cancers [11]. This thesis concentrates on pancreatic and colorectal cancers. Pancreatic cancer belongs to the five most common causes of cancer deaths, although it is not found among the five most frequently diagnosed cancer types [2]. Twenty-seven years ago, R.C. Williamson, professor and director of surgery at London's Hammersmith Hospital, referred to pancreatic cancer as "the greatest oncological challenge" [12]. And instead of improving, the situation is worsening [13]. In Europe, pancreatic cancer is the only major neoplasm showing unfavorable trends in both sexes [3]. Rahib *et al.* projected recently, that total deaths due to pancreatic cancer will increase dramatically to become the second leading cause of cancer-related deaths in the United States before the year 2030 [14].

The most frequent form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), for which the 5-year overall survival ranges from 13.6 % in stage IA to only 0.7 % in stage IV. This is the worst 5-year survival time of all common types of cancer [15]. A Finnish study reported that more than 50 % of patients with >5-year survival were wrongly diagnosed with PDAC, leading to the suggestion that even the widely published poor survival statistics may be an overestimate [15, 16].

One established risk factor of pancreatic cancer is chronic pancreatitis [17]. Historically, chronic pancreatitis and PDAC were regarded as unrelated diseases, as they were believed to

arise from different cells in the pancreas [18]. But recent work suggested a common origin in acinar cells [18]. Oxidative stress and the generation of reactive oxygen species (ROS) are important in the pathophysiology of chronic pancreatitis by modifying nucleic acids, lipids and proteins, resulting for example in DNA fragmentation, membrane disintegration and protein misfolding [18]. This can activate the immune system that in turn produces inflammatory cytokines and chemokines [18]. Inflammatory cytokines activate cyclooxygenase-2 (COX-2), which is upregulated in pancreatitis and pancreatic cancer [18]. Moreover, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is activated in PDAC [18].

In contrast to pancreatic cancer, mortality rates for colorectal cancer (CRC) have been decreasing, particularly for women, across Europe compared with the 1970s [3]. But despite advances in screening, surgery and chemotherapy, CRC is still the second leading cause of cancer deaths in Europe in both sexes [2, 19]. Similar to the relationship of pancreatitis and PDAC, inflammatory bowel disease (IBD) is an established risk factor for CRC (called colitis-associated cancer (CAC) in this case) [11]. In the last years, it became clear that immune cells, cytokines and other immune mediators play a significant role in initiation, promotion, progression and metastasis of colon tumorigenesis [19]. Similar mechanisms as in pancreatic cancer are involved in tumorigenesis of CAC, including the generation of gene mutations and DNA damage by ROS [20], the upregulation of COX-2 [21] and activation of NF- κ B [21].

Several factors can trigger chronic inflammation. One of them is aging, which leads to a remodeling of the innate and acquired immune system in a process called "inflamm-aging" [22, 23]. The incidence and death rates of many cancers, including pancreatic and colorectal cancer, increase with advancing age with most new cases occurring in people older than 50 [24, 25]. One of the main cytokines elevated in inflamm-aging is interleukin-6 (IL-6) [26], which also plays an important role in the development of CRC [27] and PDAC [28]. Another factor triggering chronic inflammation is smoking [29], which is a risk factor for pancreatic cancer [30] as well as for CRC [31].

But inflammation is not only a risk factor, it is also an accompanying characteristic of tumor growth [29]. Besides ROS, COX-2, NF- κ B and hypoxia-inducible factor 1 α (HIF-1 α), signal transducer and activator of transcription-3 (STAT3) was shown to be an important factor in the tumorigenesis of pancreatic cancer and CRC. STAT3 is frequently activated in human cancers, and a constitutively active form of STAT3 was driving transcription and induced cell transformation, qualifying STAT3 as a protooncogene [32]. STAT3 plays a role in a number of processes including tumor cell proliferation, survival, invasion, suppressing anti-tumor immunity, and therefore evolves as a promising therapeutic target [33, 34].

A cytokine activating STAT3 and being upregulated in pancreatic as well as in CRC is

IL-6 [35]. IL-6 is a pleiotropic cytokine involved in various processes of the immune system that can signal *via* two different mechanisms [36, 37]. Classic IL-6 signaling is initiated by binding of IL-6 to the membrane-bound IL-6 receptor (IL-6R). Subsequently, this complex associates in *cis* on the same membrane with the signal-transducing subunit glycoprotein 130 (gp130) [38]. While gp130 is ubiquitously expressed on all cells of the body, expression of the IL-6R is restricted mainly to hepatocytes and some leukocytes [38]. This effectively limits the types of cells responsive to IL-6. Yet, a soluble IL-6 receptor (sIL-6R) can be generated by protease-mediated receptor shedding or alternative splicing. This sIL-6R is able to bind to IL-6 in solution and subsequently, to interact with membrane-bound gp130. Thereby, IL-6 acts in *trans* on cells that do not express IL-6R themselves. Thus, this process was termed "trans-signaling" [38]. IL-6 trans-signaling is naturally inhibited by a soluble form of gp130 (sgp130). In healthy conditions, soluble gp130 (sgp130) is present in the blood in much higher concentrations than IL-6 and sIL-6R, thus creating a "buffer" that prevents trans-signaling [38]. However, in inflammatory conditions or cancer, the levels of IL-6 and sIL-6R can rise, and trans-signaling takes place.

The recombinant fusion protein Hyper-IL-6, generated by covalently linking IL-6 to the sIL-6R, directly initiates trans-signaling responses and is thus a great tool to experimentally analyze trans-signaling [39]. On the other hand, to study the inhibition of trans-signaling, sgp130 was dimerized by the constant portion of a human IgG1 antibody (sgp130Fc) [40].

IL-6 plays an important role in the pathophysiology of PDAC (extensively reviewed in chapter 2) and of CRC. IL-6 knockout mice developed reduced tumor growth in experimental models of CRC [41]. In another murine CRC model, IL-6 secreted by lamina propria T cells and macrophages activated STAT3 and promoted proliferation of tumor cells [42]. Moreover, in human CRC patients, elevated levels of IL-6 were reported in serum and tumor tissues [27]. These levels were correlated with tumor size, stage, metastasis and survival in CRC [27, 43]. Interestingly, the blood concentrations of IL-6 were also associated with high serum levels of carcinoembryonic antigen-related cell adhesion molecule5 (CEACAM5) [43, 44].

CEACAM5 is one of the best-known tumor-associated antigens for colorectal cancer [45, 46]. CEACAM5 was the first identified member of the family of carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), which comprises 12 proteins [47]. Of these 12 members, CEACAM1, CEACAM5 and CEACAM6 are regarded as valid clinical biomarkers and as promising therapeutic targets in colorectal and pancreatic cancer [47]. The last part of the presented work deals with CEACAM5 and CEACAM6. Although CEACAM5 was first described almost 50 years ago [45], knowledge of its signaling and function is still limited [47]. It was first described as a tumor marker, but its usefulness as a suitable biomarker for identifying CRC patients is continuously debated [47, 48, 49]. CEACAM5 is an intercel-

lular adhesion molecule interacting with other epithelial cells via CEACAM5-CEACAM5 bridges. In addition to its role as an adhesion molecule, CEACAM5 is involved in other cellular processes including the inhibition of differentiation programs, inhibition of apoptosis and anoikis, and disruption of cell polarization and tissue architecture [47]. CEACAM5 can interact with death receptor5 (DR5) leading to decreased anoikis and increased metastasis, or with transforming growth factor beta (TGF- β), which is involved in liver metastasis [47]. Heterophilic binding of CEACAM5 with CEACAM1 inhibits killing abilities of natural killer cells (NK cells) [47]. Moreover, liver-specific Kupffer macrophages can be activated by binding of CEACAM5 to a carcinoembryonic antigen receptor (CEAR), leading to pro-inflammatory cytokine production, including IL-6 [47].

CEACAM6 is overexpressed in a number of malignancies including pancreatic and colorectal cancer [50]. It was shown to be involved in adhesion, invasion, and metastasis [51] and to inhibit cell differentiation and anoikis [47]. Some studies suggested that IL-6 treatment increases the expression of CEACAM5 on some CRC cells [52, 53]. Yet, this relationship was not observed for all cell lines and another study found no significant stimulatory effect of IL-6 [54]. To the best of my knowledge, no one has examined the effect of IL-6 trans-signaling on the expression of CEACAM5 and CEACAM6.

1.3 Anti-inflammatory cancer therapies

In the last years it became obvious that it is not sufficient to target only the tumor cells, because the microenvironment also plays a major role for malignant initiation and progression. Therefore, therapies targeting the microenvironment are in the focus of research. One of these strategies are anti-inflammatory therapies. Grivennikov *et al.* suggested that inflammation can be targeted in several different ways: (1) Inhibition of signal transducers and transcription factors mediating survival and growth of malignant cells in response to inflammatory cytokines, (2) sequestration of cytokines and chemokines that recruit and sustain inflammatory cells, (3) reducing the inflammation following anticancer therapy, (4) specific depletion of immune and inflammatory cells that promote tumor development and progression, (5) selective inhibition of tumor-promoting cytokines [29]. Attractive targets for the first strategy are NF- κ B and STAT3 [29]. Yet, prolonged inhibition of NF- κ B can lead to a severe immunodeficiency and may enhance acute inflammation. Because of such complications, clinical translation remains a challenge [29].

None of the complications associated with NF- κ B inhibition have been reported for STAT3 inhibitors [29]. Specific inhibitors targeting phosphorylation, the disruption of the protein-protein interactions or the DNA-binding activity of STAT3 are in the focus of research, as STAT3 is activated by dimerization and binds DNA to perform its functions [55].

But even after a decade of preclinical evaluation of STAT3 inhibitors, only few of them have been used clinically [35, 55].

An even more specific strategy with possibly fewer complications is the development of drugs inhibiting receptor binding of pro-tumorigenic cytokines [29] that act upstream of transcription factors like STAT3. IL-6 evolves as a promising target as it is involved in the tumorigenesis and progression of many cancers [35]. IL-6 neutralizing antibodies showed good anti-tumor efficacy in a number of studies [35]. The specific inhibition of trans-signaling may be an even better strategy, as it probably has less side effects and was shown to be effective in a number of animal models of cancer [35].

1.4 Aims of this thesis

This thesis describes the results of two projects, both dealing with the role of IL-6 in tumor progression. The specific aims in the first part dealing with pancreatic cancer were to:

- comprehensively review the literature to summarize what had previously been described about IL-6 and pancreatic cancer
- analyze, whether pancreatic cancer cell lines express key components of the IL-6 signaling pathway
- examine the effect of IL-6 classic and trans-signaling on PDAC cells *in vitro*
- analyze, whether inhibition of IL-6 classic and trans-signaling leads to a reduced tumor growth of pancreatic cancer *in vivo*.

The second project rather focused on colorectal cancer cell lines *in vitro*. The specific aims were to:

- better understand the expression of CEACAM5 and CEACAM6 in colorectal cancer cells
- examine the impact of IL-6 classic and trans-signaling on the CEACAM5 and CEACAM6 expression of colorectal cancer cells.

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Chapter 2

Interleukin-6: A villain in the drama of pancreatic cancer development and progression

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Abstract

BACKGROUND: PDAC is a devastating malignancy with a poor prognosis and little treatment options. The development and progression of the disease is fostered by inflammatory cells and cytokines. One of these cytokines is interleukin-6 (IL-6), which plays an important role in a wide range of biologic activities. **DATA SOURCES:** A systematic search of PubMed was performed to identify relevant studies using keywords such as interleukin-6, inflammatory cytokines, inflammation and pancreatic cancer or PDAC. Articles related to IL-6 and pancreatic cancer were systematically reviewed. **RESULTS:** IL-6 is elevated in the serum of pancreatic cancer patients and correlates with cachexia, advanced tumor stage and poor survival. Its expression is enhanced by hypoxia and proteins involved in pancreatic cancer development like Kras, mesothelin or ZIP4. IL-6 in turn contributes to the generation of a pro-tumorigenic microenvironment and is probably involved in angiogenesis and metastasis. In experimental mouse models of PDAC, IL-6 was important for the development and progression of precursor lesions. **CONCLUSION:** IL-6 emerges as a key player in pancreatic cancer development and progression and hence should be considered as a new therapeutic target.

2.1 Introduction

Pancreatic cancer is presently the fourth most common cause of cancer-related death in Europe and the only major cancer type for which no improvement in mortality rates is predicted [1]. In 2012, 103,800 Europeans were estimated to be diagnosed with pancreatic cancer and 104,500 to die from it [2]. Due to the paucity of clinical signs, late diagnosis is common, and most patients present with locally advanced or metastatic disease [3]. Thus, only a few pancreatic cancer cases are diagnosed early enough to be eligible for surgery, which offers a chance of cure. The treatment for patients with advanced disease is only palliative, focusing on managing symptoms and relieving pain and suffering. Although a number of targeted therapies have proven to be beneficial in other malignancies, most targeted therapies show no clinical benefit in pancreatic ductal adenocarcinoma (PDAC) [3, 4, 5, 6].

One hallmark of PDAC is the presence of dense desmoplastic stroma [7, 8]. It comprises various cell types, including several inflammatory cell populations that lead to an immunosuppressive milieu and, lastly, to immune privilege of the tumor [9, 10, 11, 12]. Moreover, different inflammatory cytokines have been associated with PDAC development [13]. One of them is the multifunctional molecule interleukin 6 (IL-6). The present review is to highlight the role of IL-6 in pancreatic cancer development and progression, and to discuss the inhibition of IL-6 signaling as a new therapeutic strategy for pancreatic cancer.

2.2 Inflammatory cytokines contributing to pancreatic cancer development

2.2.1 PDAC: A fatal malignancy with poor prognosis

Pancreatic cancer is one of the deadliest human malignancies with incidence rates equaling mortality rates. Ninety-five percent of all pancreatic cancers are exocrine tumors of the pancreas, in particular PDAC [14]. PDAC is preceded by precursor lesions, of which the most common are known as pancreatic intraepithelial neoplasia (PanIN). PanIN lesions show a high incidence of Kras mutations, and mutations of this oncogene are also a hallmark of PDAC. Kras regulates cell proliferation, survival, differentiation, migration and extracellular communication. Oncogenic Kras drives the formation and progression of pancreatic cancer precursor lesions and is required for the maintenance of invasive and metastatic disease [15, 16].

PDAC is characterized by high mortality, rapid progression, and resistance to chemo- and radiation therapy [17]. Thus, the prognosis is extremely poor with a median survival of 24.1 months for the earliest stage and 4.5 months for the most advanced stage. The mean five-year survival rate is only approximately 6% [3]. Radical surgical resection is considered to be the only curative option. However, only 10%-20% of all pancreatic cancer patients are candidates for resection because of extensive local disease or metastatic spread at presentation,

and 80 % of patients undergoing resection still develop a recurrent tumor [18]. Searching for new treatment options of PDAC therefore remains inevitable [19].

2.2.2 Inflammation fostering PDAC development

The histological hallmark of PDAC is a dense stroma surrounding the malignant epithelial cells, which is referred to as desmoplastic reaction. It consists of various cell types as well as of acellular constituents. Extracellular matrix (ECM), enzymes, growth factors and cytokines compose the acellular compartment. The cellular components include fibroblasts, stellate, endothelial, endocrine, nerve and immune cells [20, 10]. This microenvironment provides a milieu favoring the proliferation of malignant cells and closely resembles the fibrotic stroma seen in chronic pancreatitis [13]. Chronic pancreatitis is a well-known risk factor for PDAC. Therefore, a link between inflammation and the development of pancreatic cancer has long been recognized [21, 22, 23].

PDAC development can be accelerated by cerulein-induced pancreatitis in genetically modified mouse models [24, 25], and inflammation promotes epithelial-mesenchymal transition (EMT), invasiveness and dissemination in these models [26]. A marker of systemic inflammation, C-reactive protein (CRP), is a negative prognosticator in PDAC following resection [11]. Furthermore activation of cyclooxygenase-2 (COX-2), nitric oxide synthetase and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), free radical oxygen formation and the production of the cytokines IL-1, IL-6, IL-8 and tumor necrosis factor-alpha (TNF- α) are associated with PDAC progression [11]. A COX-2 inhibitor showed a significant delay in PanIN progression in a mouse model [27]. However, a phase II study of the COX-2 inhibitor celecoxib in addition to gemcitabine and cisplatin showed no benefit over chemotherapy with gemcitabine alone [28].

Inflammation is not only a precursor of PDAC development, but also a consequence of pancreatic cancer. Initiating Kras mutations induce the upregulation of inflammatory pathways. Furthermore, the oncogenic transcription factor c-myc, which is activated in many malignancies, remodels the tumor microenvironment via stimulation of inflammatory cells and cytokine production [11]. One pathway constitutively activated in PDAC is the NF- κ B cascade. The transcription factor NF- κ B contributes to the regulation of apoptosis and oncogenesis and hence facilitates tumor growth and metastasis [29, 13]. Moreover, it regulates the expression of genes involved in inflammation by inducing the production of several inflammatory cytokines like IL-1 β , IL-8 and TNF- α . These cytokines as well as other death ligands have been shown to increasingly enhance pro-inflammatory mechanisms resulting in invasive growth and metastases in PDAC [17].

TNF- α acts like a double-edged sword in PDAC. On one hand, it has a tumor-promoting

role. Elevated serum levels of TNF- α have been found in PDAC patients and were associated with weight loss [13]. Our group observed that treatment with TNF- α increased the invasiveness of human pancreatic cancer cells *in vitro* and enhanced tumor growth as well as metastasis in mice bearing orthotopically inoculated human PDAC cells [30]. Accordingly, we found a significant reduction in tumor growth and metastasis after anti-TNF- α therapy with infliximab and etanercept [30]. Still, a recent phase I/II study using etanercept in combination with gemcitabine failed to show any significant survival improvement compared to gemcitabine alone in humans [31]. On the other hand, TNF- α has toxic effects on tumor cells in high doses. A phase I/II clinical trial showed promising results of intratumoral gene therapy with an adenoviral vector that expresses TNF- α inducible by chemotherapy and radiation [32]. Furthermore, an older phase I/II clinical study observed a beneficial outcome of combined TNF- α and anti-EGFR treatment in advanced pancreatic cancer patients [33]. Thus, the role of TNF- α in pancreatic cancer development is controversial, and targeted therapy using TNF- α remains challenging.

Other major pro-inflammatory cytokines involved in PDAC are IL-8 and IL-6 [13]. IL-8 mainly promotes angiogenesis, but it also activates the mitogen-activated protein kinase (MAPK) pathway and fosters tumor aggressiveness and invasiveness [34, 35]. IL-6 has a multifunctional role in the development and progression of pancreatic cancer by directly affecting the tumor cells as well as by modulating the tumor microenvironment [13]. Its manifold effects on pancreatic cancer development and progression are described in detail in this review.

2.2.3 Classic and trans-signaling of the pro-tumorigenic cytokine IL-6

IL-6 is a pleiotropic cytokine playing an important role in a wide range of biologic processes. It is involved in host immune defense as well as in the modulation of growth and differentiation in various malignancies [36]. IL-6 can be produced by a variety of cells, including lymphocytes, monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, and some tumor cells [37]. IL-6 binds to the IL-6 receptor, which exists in a membrane-bound (mIL-6R) and soluble form (sIL-6R). Both require a dimer of the co-receptor gp130 for signal transduction. Signaling of IL-6 via the mIL-6R is called classic signaling. As the mIL-6R is expressed only by a few cell types, mainly hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes, classic signaling is restricted to these cells. Its action is mainly anti-inflammatory. The sIL-6R is mainly produced by protease-mediated ectodomain shedding from the cell surface and, to a minor extent, by alternative splicing. In contrast to most other soluble receptors, which are antagonists, the sIL-6R stabilizes IL-6 and, in this complex, acts as a potent agonist of gp130 signaling. gp130 is ubiquitously expressed on all cells in

the body. Therefore, the IL-6/sIL-6R-complex can induce signaling also in cells lacking the IL-6R. This so-called trans-signaling mainly acts in a pro-inflammatory manner and is the preferred target of therapeutic intervention in many indications [38, 39, 40]. In analogy to other soluble cytokine receptor systems, soluble gp130 (sgp130) is the natural inhibitor of trans-signaling, and high constitutive levels of sgp130 (250-400 ng/ml) in the circulation of healthy individuals prevent global trans-signaling [40].

IL-6 signaling activates the Janus kinases JAK1, JAK2, and TYK2 which leads to the phosphorylation of signal transducers and activators of transcription-1 and -3 (STAT1 and STAT3). STAT3 is an important regulator of a number of anti-apoptotic genes, and its activity is associated with tumor growth, survival, angiogenesis and metastatic processes [41]. In fact, dysregulation of STAT3 activation is so frequent in cancer that it is widely considered as an oncogene [42, 43]. In addition to the JAK-STAT3 pathway, IL-6 activates the Ras-MAPK and phosphoinositide-3-kinase (PI3K)-Akt signaling pathways which also contribute to its anti-apoptotic and tumorigenic function [44, 37]. Hence, it is not surprising that several studies revealed an association between increased serum IL-6 concentrations in patients and advanced tumor stages or short survival in various cancers like colorectal cancer, renal cell carcinoma, prostate cancer, breast cancer, multiple myeloma, non-small cell lung carcinoma and ovarian cancer [36].

2.3 A key role of IL-6 in pancreatic cancer development and progression

2.3.1 Elevated IL-6 in the serum of PDAC patients and its correlation with a poor prognosis

It is well-established that IL-6 is elevated in the serum of pancreatic cancer patients compared to healthy controls [45, 46, 47, 48, 49, 50, 51, 52, 53] and compared to patients with chronic pancreatitis [45, 51, 52]. Microarray data comparing human PDAC tissues to matched pancreas controls showed a significant upregulation of IL-6 and its co-receptor gp130 [54]. Using qPCR, Bellone *et al.* [55] found that IL-6 was overexpressed in pancreatic cancer compared with non-tumoral pancreatic tissue. In contrast, expression of the IL-6R was not significantly changed [46, 53, 54]. IL-6 was significantly increased in correlation with tumor stage [52, 45], tumor size [52, 47, 51] and the presence of lymph node and distant metastases [52, 51]. Patients with nonresectable tumors had significantly higher IL-6 serum levels than those with tumors still resectable [52]. Moreover, IL-6 serum levels correlated with worse survival of patients with pancreatic cancer [49, 55, 52] and a poor performance status [49]. A recent study [56] examined the impact of pro-inflammatory cytokines on the outcome of gemcitabine monotherapy in patients with advanced pancreatic cancer. Univari-

ate and multivariate analysis for overall and progression-free survival identified high IL-6 serum levels as an independent predictor of poor survival.

Strikingly, Mroczko *et al.* [52] reported that the measurement of IL-6 proves clinically more useful in distinguishing pancreatic cancer from healthy conditions or chronic pancreatitis than the measurement of the classic tumor markers carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) or the inflammatory molecule CRP. This is of high significance, as the diagnosis of pancreatic cancer is usually difficult and it mostly occurs too late. However, IL-6 is also elevated in a vast number of other diseases [37] and therefore not very specific in discriminating pancreatic from any other type of cancer.

Several studies [45, 49, 51, 57, 58] described higher IL-6 levels in pancreatic cancer patients suffering from cachexia. This is interesting because severe weight loss is a major side effect of pancreatic cancer [59, 60]. A polymorphism in the IL-6 gene in pancreatic cancer patients was associated with cachexia susceptibility and survival time [61]. Martignoni *et al.* [58] found a strong IL-6 immunostaining in cancer cells from cachectic patients in contrast to tissues from patients without cachexia. Furthermore, the level of IL-6 staining in hepatocytes surrounding CD68-positive liver macrophages was significantly more intense in cachectic PDAC patients than in those without cachexia [62]. Similarly, LPS-stimulated peripheral blood mononucleated cells (PBMCs) from cachectic pancreatic cancer patients produced significantly more IL-6 than those from healthy controls [57]. Therefore, cachexia seems to be mediated by a complex interaction of different cell types in cachectic PDAC patients. These findings are in accordance with studies from other malignancies, also describing an association between IL-6 and cancer cachexia [63, 64, 65, 66].

While overexpression of IL-6 in pancreatic cancer tissue is well established, the literature is controversial about the cellular source of IL-6. Lesina *et al.* [53] described macrophages as the principal IL-6 producer in an experimental mouse model of PDAC and in human tissue. In contrast, Zhang *et al.* [67] found IL-6 expression in epithelial cells, smooth muscle actin (SMA)-positive fibroblasts and immune cells in human and murine pancreatic cancer tissues. Additionally, many pancreatic cancer cell lines express IL-6 [48, 50, 68]. Incubation with conditioned medium from pancreatic cancer cells induced IL-6 expression in mouse primary pancreatic fibroblasts [67]. Furthermore, the IL-6 production of PBMCs isolated from cachectic patients could be stimulated by IL-6-expressing pancreatic cancer cells [58]. This argues against an exclusive IL-6 production by macrophages and rather supports the idea of a complex cross-talk between cancer cells and the tumor microenvironment. Collectively, a number of studies demonstrated the overexpression of IL-6 in pancreatic cancer patients and a correlation with cachexia, advanced tumor stage and poor survival.

2.3.2 IL-6 expression contributing to a pro-tumorigenic microenvironment *in vitro*

Several factors influence the expression of IL-6 in PDAC cells. One of them is mesothelin. Mesothelin is a useful biomarker for pancreatic disease [69] and became well recognized when the 15-year old Jack Andraka won both a major international science fair competition and a US Youth Award in 2012 with his idea to couple anti-mesothelin-antibodies to nanotubes as a dipstick test for pancreatic cancer [70]. Mesothelin expression correlates with IL-6 expression in pancreatic cancer cell lines and patient samples, and overexpression of mesothelin strongly enhances the IL-6 expression of cancer cells [71]. Interestingly, only mesothelin-overexpressing cells respond to treatment with exogenous IL-6 with enhanced proliferation and to IL-6 knock-down with decreased proliferation. This effect was not observed in cells with a normal mesothelin expression [71].

Another molecule influencing IL-6 expression is the receptor for advanced glycation endproducts (RAGE) [72]. Downregulation of RAGE by shRNA significantly reduced IL-6 secretion of the human cell line Panc2.03 and the murine cell line Panc02. Furthermore, RAGE promoted the IL-6-induced phosphorylation of STAT3 and its mitochondrial localization, which occurs during autophagy. Stimulation with exogenous IL-6 increased intracellular ATP production in pancreatic cancer cells in a RAGE-dependent manner, as knockdown of RAGE by shRNA significantly diminished the ATP production [72]. Another enhancer of IL-6 expression is Kras, clearly the most important player in pancreatic tumorigenesis [73]. Furthermore, the zinc transporter ZIP4, which is overexpressed in pancreatic cancer and correlates with cancer progression, increases the expression of IL-6 [74]. In addition, IL-6 expression is enhanced by hypoxia [68, 75]. miR-21, a pro-tumorigenic miRNA induced by hypoxia, was found to be involved in this process, as a decreased expression of miR-21 reduced the hypoxia-induced IL-6 expression in MiaPaCa tumor sphere cells [75]. This is of interest because PDAC is a hypoxic tumor in humans [76, 77].

Treatment with exogenous IL-6 increased the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) in some pancreatic cancer cells [78, 79, 80]. In this context, IL-6 might contribute to angiogenesis and invasion [80]. A link between IL-6 and VEGF expression was found in other malignancies [81, 82]. Furthermore, IL-6 could contribute to the generation of a pro-tumorigenic environment by enhancing the expression of the Th2 cytokines IL-10 and IL-13 as well as of IL-5, IL-7 and granulocyte macrophage colony-stimulating factor (GM-CSF) in pancreatic cancer cell lines [68]. Moreover, IL-6 enhanced the expression of neuropilin-1 [68], which is associated with increased constitutive MAPK signaling, inhibition of anoikis and chemoresistance in pancreatic cancer [83]. However, treatment with exogenous IL-6 enhanced the proliferation of only some PDAC cell lines, as it is the case with the mesothelin-expressing cells [72, 80, 84]. Taken

together, these data show that certain protein mediators which are frequently overexpressed in pancreatic cancer, as well as hypoxia, enhance the expression of IL-6. In turn, IL-6 contributes to the generation of a pro-tumorigenic microenvironment and is probably involved in angiogenesis and metastasis. Figure 2.1 summarizes the multifaceted role of IL-6.

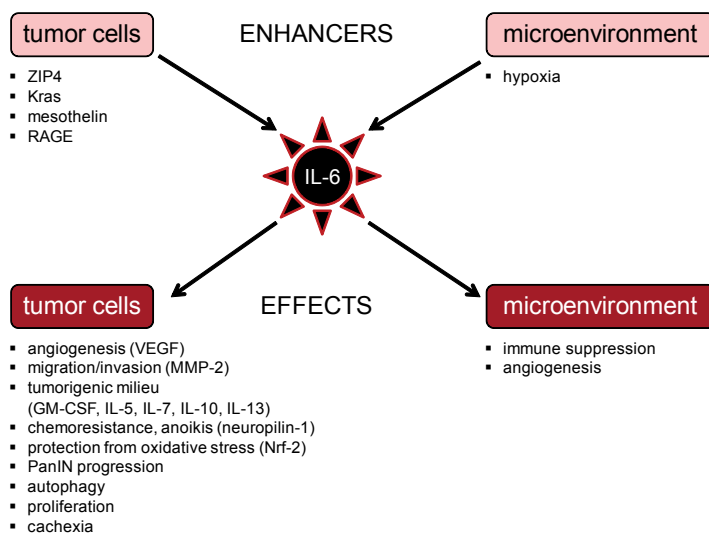


Figure 2.1: Interleukin-6 (L-6) is a key player in pancreatic cancer. Tumor cells and the tumor microenvironment (fibroblasts and immune cells) produce IL-6, which is enhanced by several factors. In turn, IL-6 acts on both the tumor cells and the microenvironment by upregulating several genes (in brackets) and activating many processes involved in cancer development and progression. **GM-CSF**, granulocyte macrophage colony-stimulating factor; **IL**, interleukin; **MMP-2**, matrix metalloproteinase-2; **Nrf2**, nuclear factor erythroid 2-related factor 2; **RAGE**, receptor for advanced glycation endproducts; **VEGF**, vascular endothelial growth factor

2.3.3 IL-6 favoring the development of precursor lesions and the progression to PDAC in mouse models

Studies analyzing the role of IL-6 *in vivo* were mainly performed in the Kras mouse model. These mice carry a latent point-mutant allele of Kras (Kras^{G12D}). Cre-mediated recombination leads to deletion of a transcriptional termination sequence and expression of the oncogenic protein. Pancreas-specific expression of Cre causes ductal lesions that recapitulate the full spectrum of human PanINs. Some of these lesions progress to invasive and metastatic adenocarcinomas [85]. Therefore, the (Kras^{G12D}) model is regarded as an appropriate system to study the initiation and early steps in pancreatic cancer development.

Lesina et al. [53] showed that IL-6 mRNA increased over time in the pancreas of Kras^{G12D} mice compared with control mice. Here, IL-6 was found to be predominantly expressed by infiltrating immune cells, especially F4/80-positive macrophages, whereas

acinar cells expressed only low levels of IL-6. *Kras*^{G12D} mice that do not express any IL-6 (*Kras*^{G12D};*IL6*^{-/-}) developed less PanINs. However, a bone marrow transplantation of *IL-6*^{+/+} cells into these mice increased the frequency of PanIN-3 lesions. Therefore, macrophage-derived IL-6 seems to be important for PanIN progression in this model. In isolated acinar cells, treatment with exogenous IL-6 induced only a weak STAT3 phosphorylation. Similarly, isolated macrophages of *Kras*^{G12D} mice were not able to stimulate STAT3 phosphorylation in these cells, even after pre-treatment with IL-6. In contrast, a fusion protein of IL-6 and the sIL-6R, termed Hyper-IL-6 [86], strongly activated STAT3 in acinar cells, suggesting a role for IL-6 trans-signaling. Moreover, (*Kras*^{G12D}) mice in which trans-signaling was inhibited by co-expression of an Fc fusion protein of the trans-signaling inhibitor sgp130 (*Kras*^{G12D};*sgp130Fc*), developed less PanINs. Thus, IL-6 trans-signaling was important for the initiation and progression of PanINs in this model [53]. However, in cell lines generated from primary tumors of the *Kras*^{G12D} mice, IL-6 alone was able to increase STAT3 phosphorylation. This suggests the occurrence of a switch towards classic IL-6 signaling at later stages in tumor development. Pancreas-specific knockout of STAT3 led to a strong reduction of PanINs. PanINs-2 and 3 were not detectable and the tumor incidence was significantly decreased [53]. A similar study published by Corcoran et al. [54] in the same year confirmed the role of STAT3 in the development of the precursor lesions acinar-to-ductal metaplasias (ADMs) and PanINs.

Zhang *et al.* [67] described similar results in the elegant *iKras** mouse model, in which the *Kras* mutation is inducible and reversible. IL-6 mRNA was elevated in *iKras** pancreata with embryonic *Kras* activation compared to wildtype controls. *iKras**;*IL-6*^{-/-} mice showed less ADMs and rare PanINs in contrast to *iKras** mice. Interestingly, pancreatitis-driven initial PanIN formation was independent of IL-6. However, the lesions which were formed in the absence of IL-6 showed reduced activation of the MAPK, Akt and STAT3 signaling pathways and had a lower proliferation rate [67]. Strikingly, pancreatitis-induced neoplastic lesions in *iKras**;*IL-6*^{-/-} mice regressed, whereas the lesions in *iKras** mice progressed over time to high-grade PanINs. In the absence of IL-6, the stroma of PanINs was remodeled, resulting in a normal pancreatic parenchyma with some adipose tissue infiltration. PanIN elimination was due to a combination of apoptotic cell death, redifferentiation of cells toward the acinar lineage and increased proliferation of acinar cells. Furthermore, IL-6 seemed to protect cancer cells from oxidative stress by upregulating nuclear factor erythroid 2-related factor 2 (Nrf2), a key player in the reactive oxygen species (ROS) detoxification pathway. In summary, this study showed that IL-6 switches the balance between tissue-repair and carcinogenesis in the pancreas [67]. Fukuda *et al.* [87] also demonstrated an upregulation of IL-6 after the induction of pancreatitis. This upregulation was greatly enhanced by the *Kras* mutation and

diminished by STAT3 knockout. This study furthermore highlights the importance of STAT3 in PDAC development [87].

In a subcutaneous tumor model of human pancreatic cancer cells, the effect of IL-6 knockdown by shRNA varied among different cell lines. While it caused necrosis in tumors from Capan-1 cells and retarded the growth of HPAC cells, it had no obvious effect on tumors from CFPac-1 cells [73]. However, subcutaneous models do not reflect the complex microenvironment of pancreatic cancer [88].

Saito et al. [84] suggested that IL-6 inhibits the development of liver metastasis of pancreatic cancer cells. However, the study described only a statistically significant difference between a clone with a high IL-6 production and a non-transfected control, while the frequency of liver metastases between the IL-6 high producer and a clone with no IL-6 secretion was not statistically significant. A clone that secreted low levels of IL-6 showed even more metastatic nodules than the non-producer. This questions the hypothesis that IL-6 favors metastasis. Furthermore, the study was performed in nude mice, which showed an enhanced humoral immune response against tumor cells overexpressing human IL-6. Therefore, it is difficult to distinguish which effects were due to an immune reaction against the cancer cells expressing foreign IL-6 and which effects could be attributed to a real physiological action of IL-6 [84].

In addition to the direct effects on cancer cells, IL-6 can act on the various stromal constituents of the tumor microenvironment. To our knowledge, no specific data for pancreatic cancer are available, but the general mechanisms including immune suppression and angiogenesis have recently been reviewed [43]. Conversely, some studies suggested a positive role of IL-6 trans-signaling in the mobilization of anti-tumor immunity to tumor tissues [43]. However, despite high IL-6 signaling in PDAC tissues these tumors are highly immunosuppressive and characterized by the presence of only a few tumor-specific CD8+ T cells, B cells and tumor-reactive antibody producing plasma cells in the tumor mass [89, 10, 20]. Thus, IL-6 trans-signaling in physiologic concentrations does not seem to acutely activate the vasculature and contribute to lymphocyte trafficking in PDAC.

In summary, IL-6 was shown to play an important role in the development and progression of precursor lesions in mouse models of PDAC. Thus, IL-6 has emerged as a new target for the treatment of PDAC [13, 14].

2.3.4 Fighting the villain: IL-6 inhibition as a new treatment option for PDAC

As IL-6 is overexpressed in many malignancies and contributes to tumor progression, blockade of IL-6 signaling could become a new treatment option. Some clinical studies targeting IL-6 in other cancers have already been performed and were recently reviewed elsewhere [37]. The chimeric anti-IL-6 monoclonal antibody siltuximab stabilized disease in

ovarian and renal cancer [37]. It furthermore led to partial or complete response in B-cell non-Hodgkin's lymphoma, multiple myeloma (MM) and Castleman's disease [37]. In castration-resistant prostate cancer, no RECIST (Response Evaluation Criteria in Solid Tumors) response was reported, but 23 % of patients showed stable disease and most patients had decreased CRP plasma levels [90]. A phase II multicentre study of siltuximab, alone or in combination with dexamethasone, for patients with relapsed or refractory MM showed no responses to siltuximab monotherapy, but a 23 % response rate for combination therapy [91]. Tocilizumab, a humanized anti-IL-6R antibody, suppressed *in vivo* growth of human oral squamous cell carcinoma in a pre-clinical model [92]. Tocilizumab is furthermore being evaluated in current open-label phase I (USA) and II (France) trials as monotherapy in MM patients [37]. Recently, a case report described the successful use of tocilizumab for the treatment of cancer cachexia [93]. The humanized aglycosylated anti-IL-6 monoclonal antibody clazakizumab was well-tolerated in a phase IIa study in the treatment of non-small cell lung cancer-related cachexia [94]. It improved anemia and the lung symptom score, diminished weight loss and reversed fatigue [94].

Despite the many preclinical studies describing the importance of IL-6 in PDAC development and progression, no clinical trials evaluating IL-6 inhibition as a treatment option in PDAC have yet been performed. Considering the devastating prognosis of PDAC patients, new therapies are urgently needed. Zhang et al. [67] showed that treatment of ten-week-old KPCY mice exhibiting PanINs with an anti-IL-6 antibody reduced the number of precursor lesions. Our group recently observed that palliative and adjuvant inhibition of trans-signaling by sgp130Fc as well as inhibition of classic signaling by tocilizumab significantly reduced orthotopic tumor growth of human PDAC cells in SCID/bg mice (manuscript in preparation¹). Thus, inhibiting IL-6 signaling might be a new targeted treatment option and should be evaluated in clinical studies. In this context, it will also be of particular interest whether a complete blockade of IL-6 signaling is necessary or whether selective blockade of trans-signaling is sufficient. Most *in vivo* models suggested trans-signaling as the main mechanism driving tumor growth. This inhibition could clinically be achieved by the sgp130Fc derivative FE 999301, which is so far the only specific trans-signaling inhibitor, and which is currently tested in phase I clinical trials. Blockade of trans-signaling should minimize possible side effects of IL-6 inhibition like the risk for bacterial infections and could therefore be a superior strategy [96, 97].

As IL-6 is strongly connected to cancer cachexia, IL-6 inhibition might not only be useful in inhibiting tumor progression but also in improving the overall health status of the patient. Moreover, IL-6 has recently been linked to major depression in pancreatic cancer

¹These data are shown in chapter 3 and were published by now [95].

patients [98]. Thus, IL-6 inhibition might generally improve the quality of life in cancer patients.

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Chapter 3

Inhibition of IL-6 signaling significantly reduces primary tumor growth and recurrences in orthotopic xenograft models of pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal human tumors, with radical surgical resection as the only curative treatment option. However, resection is only possible in a small fraction of patients, and about 80 % of the patients develop recurrences. PDAC development is facilitated by the cytokine interleukin-6 (IL-6), which acts via classic and trans-signaling. Both pathways are inhibited by the anti-IL-6-receptor antibody tocilizumab, whereas the fusion protein sgp130Fc specifically blocks trans-signaling. Here, we show that conservative or adjuvant therapy with both inhibitors reduces tumor growth in an orthotopic model of human Colo357

cells in SCID/bg mice. In the conservative setting, median primary tumor weight was reduced 2.4-fold for tocilizumab and 4.4-fold for sgp130Fc. sgp130Fc additionally led to a decrease in microvessel density, which was not observed with tocilizumab. In the adjuvant therapeutic setting after surgical resection of the primary tumor, treatment with tocilizumab or sgp130Fc decreased the local recurrence rate from 87.5 % in the control group to 62.5 or 50 %, respectively. Furthermore, the median weight of the local recurrent tumors was clearly diminished, and both inhibitors reduced the number of distant metastases. A significant reduction of tumor weight and metastases – comparable to gemcitabine treatment – was also observed with both inhibitors in another model using the poorly differentiated PancTuI cells. Our findings demonstrate the inhibition of IL-6 as a new treatment option in PDAC.

Novelty and impact of the paper: IL-6 plays a critical role in the progression of pancreatic cancer, and its inhibition may be key in the therapeutic battle against the disease. IL-6 acts through both an anti-inflammatory classic signaling pathway involving membrane-bound IL-6R and a pro-inflammatory trans-signaling pathway involving soluble IL-6R and gp130. Using a clinically adapted xenotransplant model, this study shows that inhibition of both pathways, with either tocilizumab or sgp130Fc, which specifically blocks transsignaling, leads to significant reductions in human pancreatic tumor growth. Following surgical resection of primary tumors, the inhibitors decreased local tumor recurrence rates and reduced numbers of distant metastases.

Key words: IL-6, PDAC, tocilizumab, sgp130Fc, inflammation

Abbreviations: ADM: acinar-to-ductal metaplasia; ANOVA: analysis of variance; DMSO: dimethyl sulfoxide; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; FFPE: formalin-fixed and paraffin-embedded; IL-6: interleukin-6; IL-6R: IL-6 receptor; i.p.: intraperitoneal; mIL-6R: membrane-bound form of the IL-6R; PanIN: pancreatic intraepithelial neoplasia; PBS: phosphate-buffered saline; PDAC: pancreatic ductal adenocarcinoma; PMA: phorbol-12-myristate-13-acetate; RT-PCR: reverse transcriptase polymerase chain reaction; RIPA: radioimmunoprecipitation assay; RT: room temperature; SCID/bg: severe combined immunodeficient beige; sIL-6R: soluble IL-6 receptor; STAT3: signal transducer and activator of transcription-3; TBS: tris-buffered saline

3.1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal human tumors with a five-year survival rate of approximately 6% [1]. Radical surgical resection is considered to be the only curative option. However, only 10-20% of all pancreatic cancer patients are candidates for resection and 80% of all patients treated in this curative manner develop a recurrent tumor [2]. Moreover, PDAC is projected to be the second leading cause of cancer-related death in 2030 in the United States [3]. Searching for new treatment options of PDAC therefore remains an urgent task.

One of the risk factors for the development of PDAC is chronic pancreatitis [4, 5]. In the last years, it became obvious that inflammation is a hallmark of cancer and that chronic inflammation favors the development of tumors in many cases [6]. The tumor-associated inflammation forms a complex network, in which the involved cells and cytokines can develop both pro and anti-tumorigenic effects [7, 8].

One well-known pro-tumorigenic molecule is the pleiotropic cytokine IL-6. Its concentration in the serum of cancer patients is often elevated compared to healthy controls, and these elevated levels are correlated with a poor prognosis [9]. IL-6 signals via two distinct mechanisms, which both require a dimer of the co-receptor gp130 for signal transduction. The so-called classic signaling employs a membrane-bound form of the IL-6R (mIL-6R) and mainly mediates anti-inflammatory effects. Additionally, the IL-6R exists in a soluble form (sIL-6R), which is produced either by proteolytic cleavage (shedding) from the cell surface or by alternative splicing. In contrast to many other soluble receptors, the sIL-6R does not inhibit IL-6. Instead, it acts as a stabilizer and, in the form of the IL-6/sIL-6R complex, as a potent agonist of gp130 signaling. As gp130 is expressed on all cells of the body, IL-6 can thereby induce signaling also in cells lacking the IL-6R. This so-called trans-signaling mainly acts in a pro-inflammatory way and is the preferred target of therapeutic intervention in many indications. Soluble gp130 (sgp130) is the natural inhibitor of trans-signaling [10, 11, 12].

Several studies proved a role of IL-6 in pancreatic cancer (recently reviewed in Refs. [13] and [14]). Lesina *et al.* showed that activation of signal transducer and activator of transcription-3 (STAT3) by IL-6 trans-signaling is required for the progression of the earliest premalignant pancreatic lesions, pancreatic intraepithelial neoplasias (PanINs), and the development of PDAC in a mouse model of Kras-driven disease [15]. Corcoran *et al.* confirmed the importance of STAT3 for the development of PanINs and acinar-to-ductal metaplasia (ADM). They also found a significantly higher expression of gp130 and IL-6 in microarrays of human PDACs compared to matched normal pancreata [16]. A more recent publication highlights IL-6 as a potential therapeutic target, as it is a key player in the maintenance of PanINs and the progression to cancer [17]. However, these *in vivo* studies used a purely

murine model of pancreatic cancer, and little is known about the impact of IL-6 on human PDAC cells *in vivo*.

Inhibiting IL-6 signaling might be a new therapeutic option for PDAC. One possibility would be the treatment with the humanized monoclonal anti-IL-6R antibody tocilizumab. This antibody blocks both classic and trans-signaling and is already approved for the treatment of rheumatoid arthritis, juvenile idiopathic arthritis and Castleman's disease [11]. Its potential therapeutic effect on PDAC, however, has not yet been examined. A more selective alternative to tocilizumab is the designer molecule sgp130Fc, which was constructed by fusing a human IgG1-Fc part with two gp130 extracellular domains. Similar to sgp130 but with much higher efficacy, sgp130Fc specifically inhibits IL-6 trans-signaling without affecting classic signaling, and an optimized sgp130Fc variant is currently in clinical development [11, 18, 19]. The aim of our study was to evaluate the therapeutic efficacy of tocilizumab and sgp130Fc on cancer growth of human PDAC cells *in vivo*. We used a conservative and an adjuvant treatment setting in an orthotopic xenograft model of human Colo357 and PancTuI cells in SCID/bg mice to closely simulate both clinical situations. We discovered that both treatments reduced tumor size, as well as local and distant recurrence rate.

3.2 Materials and Methods

3.2.1 Cell culture and proteins

BxPC3 and Panc1 cells were obtained from ATCC (LGC Standards, Wesel, Germany). Panc89 cells were a kind gift of Dr. T. Okabe (Tokyo, Japan), PancTuI cells of Dr. M. von Bülow (Mainz, Germany) and Colo357 cells of Dr. R. Morgan (Denver, CO) [20]. These cells were routinely cultured in RPMI-1640 medium (Gibco/Life Technologies, Darmstadt, Germany) supplemented with 10 % fetal bovine serum (FBS, PAN-Biotech, Aidenbach, Germany), 1 mM sodium pyruvate (Gibco) and 2 mM glutaMAX (Gibco). The E6/E7-HPV16-immortalized human pancreatic ductal epithelial cell line HPDE was a gift of Dr. M. Tsao (Toronto, Canada) [21, 22] and was routinely cultured in HPDE medium (RPMI 1640 medium supplemented with 10 % FBS/ 2 mM glutaMAX and mixed immediately before use 1:1 with keratinocyte medium SFM (Gibco) supplemented with 0.025 % bovine pituitary extract/ 2.5 µg/l epidermal growth factor (Gibco). All cells were maintained at 37 °C in a humid atmosphere with 5 % CO₂ and routinely checked for mycoplasma contamination with the MycoTrace kit (PAA/GE Healthcare, Cölbe, Germany).

IL-6 and Hyper-IL-6 were produced by the group of Stefan Rose-John as previously described [23, 24]. Tocilizumab (ACTEMRA®/RoACTEMRA®) was obtained from Roche. sgp130Fc was produced by CONARIS Research Institute AG, Kiel as previously described [19].

3.2.2 Laboratory animals

Four-week-old female severe combined immunodeficient beige (SCID/bg) mice were obtained from Charles River (Sulzfeld, Germany). They were housed in a sterile environment and allowed to acclimatize for ten days. Animal experiments and care were in accordance with the guidelines of institutional authorities and approved by local authorities (number V232-7224.121-7(13-1/12) and V312-7224.121-7(123-10/11)).

3.2.3 Orthotopic xenograft transplantation and treatment in the conservative setting

Orthotopic injection was performed as previously described [25]. In brief, Colo357 cells or PancTuI cells were trypsinized and suspended in Matrigel™ (BD Bioscience, Heidelberg, Germany) at a concentration of 4×10^7 cells/ml and stored on ice. After median laparotomy, 25 μ l of the tumor cell suspension containing 1×10^6 tumor cells were injected carefully into the tail of the pancreas. After acclimatization, mice were randomly assigned to the treatment groups. Treatment *via* intraperitoneal (i.p.) injection started three days after tumor cell inoculation and was repeated twice a week. For Colo357 cells, all mice survived the surgery. Two groups received the inhibitor sgp130Fc either in a low (0.5 mg/kg) or a high concentration (2.5 mg/kg). Tocilizumab was administered to two other groups in doses equimolar to those used for sgp130Fc (0.4 mg/kg or 2.0 mg/kg, respectively). The control group received phosphate-buffered saline (PBS), the solvent for the inhibitors. All mice were inspected daily for complications, and tumor growth was monitored by weekly ultrasound imaging. Thirty-five days after tumor cell injection, the mice were sacrificed.

In the PancTuI model, four mice died in the first two days after surgery, and one mice died later during ultrasound imaging. Mice were divided into six groups and received either PBS, sgp130Fc (0.5 mg/kg), tocilizumab (0.4 mg/kg), gemcitabine (1 mg/kg), a combination of gemcitabine (1 mg/kg) and sgp130Fc (0.5 mg/kg) or a combination of gemcitabine (1 mg/kg) and tocilizumab (0.5 mg/kg). Tumor growth was monitored by weekly ultrasound imaging, and the mice were sacrificed on day 28 after tumor cell inoculation. Tumors were removed and weighed. Subsequently, the tumor was cut into two equal parts, which were immediately either frozen in liquid nitrogen or formalin-fixed and later paraffin-embedded (FFPE). The mice were carefully observed for any macroscopic metastases in liver, spleen, and mesenterium.

3.2.4 Ultrasound imaging

The ultrasound device (Vevo 2100, VisualSonics, Toronto, Canada) was equipped with a 40 MHz transducer, and a 3D motor on a rail system for automated acquisition of 3D stacks. The transducer position, gain settings, and midfield focus were initially optimized and maintained throughout each experiment. The transducer was fixed on the rail system, and placed

centrally above the tumor. Three-dimensional images in the B mode were acquired by computer controlled linear translation of the transducer over the whole length of the tumor. Two-dimensional images were acquired every 100 μm for morphologic analysis.

3.2.5 Relaparotomy, tumor resection and treatment in the adjuvant setting

Ten days after tumor cell inoculation, the tumors were resected as described before in detail [26]. Treatment of the mice started three days after the resection. Mice were randomly assigned to three groups, and PBS, sgp130Fc (0.5 mg/kg) or tocilizumab (0.4 mg/kg) were injected i.p. twice a week. Tumor growth was monitored by ultrasound as described above, and animals were sacrificed on the 35th postoperative day. Organs and tumors were examined as described in the conservative setting.

3.2.6 Analysis of FFPE samples by immunohistochemistry

Sections (2 μm) of FFPE samples were mounted onto slides, deparaffinated with xylene and rehydrated in descending concentrations of ethanol. Hematoxylin and eosin (HE), pancytokeratin (KL1, IMMUNOTECH SAS/Beckman Coulter, Marseille, France) and Ki67 (Dako, Glostrup, Denmark) staining was performed using the autostainer BondTM Max System (Leica-Menarini, Berlin, Germany). For CD31 staining, antigen retrieval was performed in citrate buffer (pH 6) using the Pascal Pressure Cooker (Dako). All washing steps were performed at room temperature (RT) with tris-buffered saline (TBS). Blocking was performed as previously described [27]. Slides were incubated with primary antibody overnight at 4 °C (SZ31, Dianova, Hamburg, Germany; 1:30 dilution in Antibody Diluent, Zytomed, Bargteheide, Germany). Detection and counterstaining with hemalum were performed as previously described [27]. Slides were scanned and analyzed with Scn400 Image Viewer software (Leica, Solms, Germany). At a 10x magnification, the tumors were screened for vascular hot spots, which are the areas with the most CD31+ structures. These areas were further analyzed at a 30 magnification. A screenshot was taken and all clearly separate CD31+ structures per picture were counted using the ImageJ software (National Institute of Health, Bethesda, MD). The mean of all CD31+ structures per field in three to five hot spots per tumor was calculated. Five different tumors of each group were evaluated.

3.2.7 Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated with RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured in a Nanodrop spectrophotometer (Thermo Scientific) and quality-checked on a 1 % agarose gel. 2 μg of RNA was reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). PCR was performed using the Dream Taq Green Polymerase (Thermo Fisher Scientific). The primer sequences are

depicted in table 1, and the following conditions were used: initial denaturation: 95 °C, 2 min; denaturation: 95 °C, 30 sec; annealing: 60 °C, 30 sec; extension: 72 °C, 1 min (40 cycles); final extension: 72 °C, 10 min. The PCR product was analyzed by agarose gel electrophoresis.

name	sequence 5'-3'	amplicon [bp] (mRNA/genomic)	reference
hu_mIL6R_For	CATTGCCAATGTTCTGAGGTTTC	280/not amplified	[28]
hu_sIL6R_For	GCGACAAGCCTCCCAGGTTTC	278/not amplified	[28]
hu_IL6R_Rev	GTGCCACCCAGCCAGCTATC	278(mIL6R),280(sIL6R)/not amplified	[28]
hu_gp130C	GGTACGAATGGCAGCATAACA	713/10170	[29]
hu_gp130D	CTGGACTGGATTCATGCTGA	713/10170	[29]
hu_IL6_For1	TCCACAAGCGCCTTCGGTCC	621/not amplified	primerblast
hu_IL6_Rev1	TTGCCGAAGAGCCCTCAGGCT	621/not amplified	primerblast
hu_RPL22_For	TCGCTCACCTCCCTTCTAA	250/6652	[30]
hu_RPL22_Rev	TCACGGTGATCTTGCTCTTG	250/6652	[30]

Table 3.1: Primer sequences used for RT-PCR

3.2.8 Phorbol-12-myristate-13-acetate (PMA) stimulation and enzyme-linked immunosorbent assays (ELISAs)

For determination of endogenous protein production, Colo357 cells were seeded in 6-well plates. After 24 h, the medium was replaced or changed to serum-free medium. Twenty-four hours, 48 h and 72 h later, the supernatants were collected and centrifuged for 15 min at 16,000 x g and 4 °C in a benchtop centrifuge to remove cells and cellular debris. The purified supernatants were stored at -80 °C until ELISA analysis. For PMA stimulation, Colo357 cells were seeded in a 96-well plate. On the next day, the medium was changed to remove non-adherent or dead cells. After 72 h, the supernatants were collected to measure the baseline (unstimulated) sIL-6R production of the cells (data not shown). Subsequently, the medium was changed, and the cells were stimulated for 2 h at RT with medium containing either 100 nM PMA (Calbiochem/Merck, Darmstadt, Germany) dissolved in dimethyl sulfoxide (DMSO) or 0.5 % DMSO as solvent control in triplicate wells. Supernatants from the triplicate wells were harvested and processed as described above. IL-6 and sIL-6R concentrations were measured using ELISA kits (R&D Duoset, R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

3.2.9 Preparation of cell lysates and western blots

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer, sonicated and centrifuged (13,000 rpm, 15 min, 4 °C) to remove cellular debris. Protein concentration was determined with the DC assay (Bio-Rad Laboratories, Munich, Germany). Equal amounts of protein were loaded onto a 4-20 % tris-glycine gel (Life Technologies) and separated by SDS-PAGE. Proteins were blotted on a PVDF membrane (Immobilon-FL; Millipore/Merck, Darmstadt, Germany), blocked with 5 % milk or bovine serum albumin for 1 h at RT and in-

cubated with the primary antibody overnight at 4 °C. Secondary antibody incubation was 1 h at RT. All washes were performed with TBS supplemented with 0.01 % Tween-20. Blots were dried with methanol and scanned in an Odyssey imager (LI-COR, Bad Homburg, Germany). The following antibodies were used: P-STAT3 (#9131, Cell Signaling Technology/New England Biolabs, Schwalbach, Germany), STAT3 (#9139, Cell Signaling Technology), β -actin (ab6276, Abcam, Cambridge, UK), goat-anti-mouse-IRDye680 (LI-COR) and goat-anti-rabbit-IRDye800CW (LI-COR).

3.2.10 AlamarBlue test

Colo357 cells were seeded in 96 well plates. After 24 h the cells were serum starved over night. The next day cells were treated with IL-6, Hyper-IL-6, sgp130Fc or Tocilizumab. After 72 h, 10 μ l alamarBlue[®] (Invitrogen/Life Technologies) was added to the supernatants. Three hours later the emission at 595 nm was measured in the SPECTRA Fluor Plus fluorescence reader (Tecan, Maennedorf, Switzerland) with excitation at 550 nm. Five replicate wells were measured per treatment.

3.2.11 Statistics

Tumor weights were first analyzed for normal distribution with the Shapiro-Wilk normality test and for equal variance with the modified robust Brown-Forsythe Levene-type test. Subsequently, an analysis of variance (ANOVA) test was performed for normally distributed data and a Kruskal-Wallis rank sum test for non-normal data. Pairwise comparisons using pairwise t-tests or the Wilcoxon rank sum test followed as post-hoc tests. Multiple testing correction was performed using the p-adjustment method suggested by Benjamini and Hochberg [31]. All analyses were performed with the R statistical software (<http://www.r-project.org>). The same procedure was performed for the proportion of Ki67+ cells. For CD31 analysis, p-values were calculated with the Tukeys multiple comparison test after an ANOVA test using the Graphpad Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA).

3.3 Results

3.3.1 Human pancreatic cancer cells express IL-6 as well as the membrane-bound and soluble IL-6R.

In the first set of experiments, we investigated the expression of IL-6, mIL-6R and sIL-6R in different human pancreatic cancer cell lines at the mRNA level. Interestingly, IL-6 mRNA was found in all cell lines, including the cell line HPDE (E6/E7-HPV16-immortalized human normal pancreatic ductal epithelial cells) (Fig.3.1 A). The same observation was made for the mIL-6R and the alternatively spliced isoform that encodes the sIL-6R. As expected, the co-receptor gp130 was also expressed by all cell lines. Therefore, all molecules for IL-6

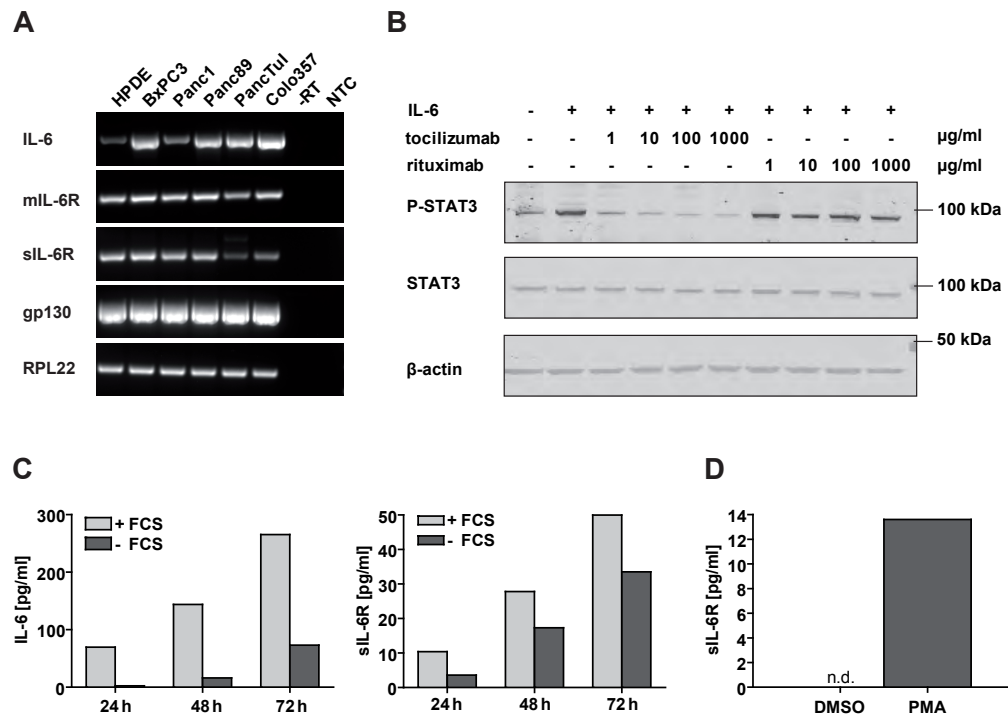


Figure 3.1: IL-6, mIL-6R, sIL-6R and gp130 are expressed in human pancreatic cell lines. **A**, RT-PCR was performed with primers for IL-6, mIL-6R, sIL-6R and gp130 after RNA isolation from human pancreatic cancer cells cultured in vitro. Transcripts of the ribosomal protein RPL22 were analyzed to ensure equal cDNA content. NTC, no template control; -RT, negative control without reverse transcriptase. **B**, Cells were serum-starved overnight and pre-incubated with tocilizumab (or rituximab as an isotype control) for 1 h. Subsequently, cells were treated with IL-6 [100 ng/ml] for 15 min. Cell lysates were analyzed for STAT3 phosphorylation in Western blot. Protein levels of β-actin were analyzed to ensure equal protein content. **C**, Endogenous production of IL-6 and sIL-6R by Colo357 cells was analyzed in cell culture supernatants. Cells were cultured with or without FBS for different periods. Supernatants were collected and analyzed by ELISA. **D**, Colo357 cells were stimulated with 100 nM PMA for 2 h. Incubation with the PMA solvent DMSO was used as a negative control. Supernatants were collected, and shed IL-6R was detected by ELISA. n.d., not detectable.

signaling were expressed in 5/5 human pancreatic cancer cell lines. For further analysis, we selected the Colo357 cells because these had a particularly high IL-6 expression (as determined by previous studies).

To confirm the expression of the functional receptor complex on the cell surface, Colo357 cells were incubated with exogenous IL-6. This stimulation increased the downstream phosphorylation of STAT3, which could be decreased by the anti-IL-6R antibody tocilizumab (Fig.3.1 B). Thus, Colo357 cells express the functional IL-6R signaling module of mIL-6R and gp130 on their surface. Furthermore, endogenous production of IL-6 was confirmed by ELISA analysis of the supernatants of Colo357 cells after 24, 48 and 72 h. IL-6 accumulated over time. The sIL-6R was also present in the supernatants at low concentrations (Fig.3.1 C).

To investigate whether this sIL-6R was produced by alternative splicing, protease-mediated receptor shedding, or a combination of both, we used an indirect approach. IL-6R is mainly shed by ADAM17, which is strongly activated by phorbol-12-myristate-13-acetate (PMA) [32]. If mIL-6R and ADAM17 are present on a cell surface, short term PMA treatment will increase the concentration of sIL-6R in the supernatant. We performed a PMA stimulation 72,h after seeding of the cells, when sIL-6R had already accumulated in the supernatant. The supernatant was replaced either with PMA- or DMSO-containing medium, and cells were incubated for 2 h. PMA-treated cells exhibited sIL-6R in the supernatant, while no sIL-6R was detected after control incubation with the PMA solvent DMSO (Fig.3.1 D). This result shows that Colo357 cells can produce sIL-6R not only by alternative splicing (Fig.3.1 A) but also by shedding (Fig.3.1 D). Taken together, these results demonstrate that Colo357 cells are suitable as a model for the inhibition of IL-6 signaling, as they feature all necessary signaling molecules and functions.

3.3.2 The *in vitro* proliferation of Colo357 cells is not influenced by IL-6, Hyper-IL-6, sgp130Fc or tocilizumab.

Tumor growth largely depends on the proliferation of cancer cells. Therefore, we examined whether IL-6 influences cell proliferation. Stimulation with exogenous IL-6 or Hyper-IL-6, which is a fusion protein of IL-6 and the sIL-6R and mimicks the IL-6 trans-signaling complex [24], did not change the proliferation of Colo357 cells *in vitro* as indirectly analyzed by metabolic activity measurement in an alamarBlue[®] test (Fig.3.2). Furthermore, no difference was observed after complete inhibition of endogenous IL-6 signaling by tocilizumab or inhibition of endogenous IL-6 trans-signaling by sgp130Fc (Fig.3.2). Therefore, IL-6 signaling does not directly influence the proliferation of Colo357 cells under the tested *in vitro* con-

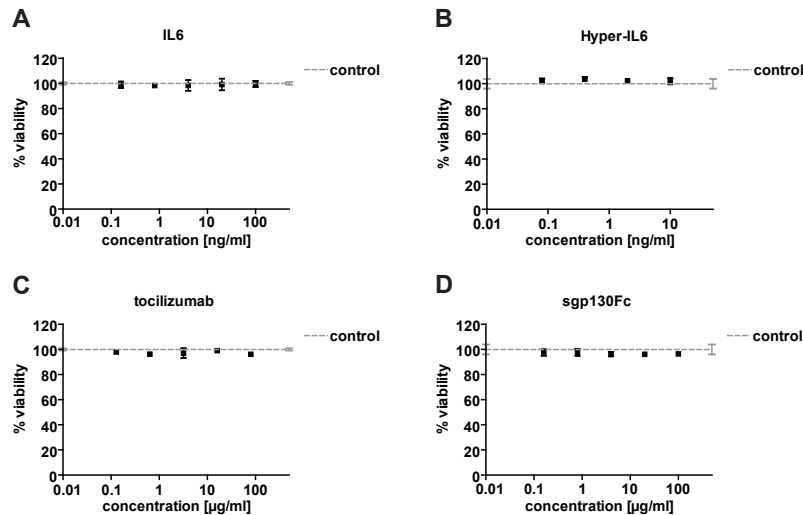


Figure 3.2: Treatment with IL-6, Hyper-IL-6, sgp130Fc or tocilizumab did not change the proliferation of Colo357 cells *in vitro*. Colo357 cells were seeded in a 96-well plate, serum-starved over night and treated with IL-6 or Hyper-IL-6 in different concentrations in serum-free medium. Alternatively, endogenous IL-6 (trans-)signaling was blocked by sgp130Fc or tocilizumab. After 72 h, alamarBlue solution was added to the medium and the emission at 595 nm was measured three hours later. Cells treated with serum-free medium were used as control (100 %). Five replicate wells were used for each condition. Data shown represent means and standard deviation.

ditions. Moreover, clonogenic growth of single Colo357 cells was not significantly changed after treatment with the stimuli or inhibitors (data not shown).

3.3.3 Conservative treatment with sgp130Fc or tocilizumab significantly decreases primary tumor growth of Colo357 cells *in vivo*.

To analyze the pharmacological effect of the inhibitors tocilizumab and sgp130Fc *in vivo*, we treated mice inoculated with Colo357 cells with different concentrations of the inhibitors (see scheme in Fig. 3.3A). A remarkable decrease in tumor weight was observed in mice treated with sgp130Fc or with tocilizumab compared to PBS injection in this conservative treatment setting (Fig. 3.3 B). Representative ultrasound images of day 34 after tumor cell inoculation are shown in Fig. 3.3 C. Each inhibitor was applied at two different concentrations. Interestingly, the lower doses had a stronger effect. A similar inverse dose response was observed before in a model of inflammation-associated colon cancer [11]. The smallest tumors were found in mice that were treated with a sgp130Fc dose of 0.5 mg/kg bodyweight. Only very few mice showed distant metastases with no significant differences between the groups (data not shown).

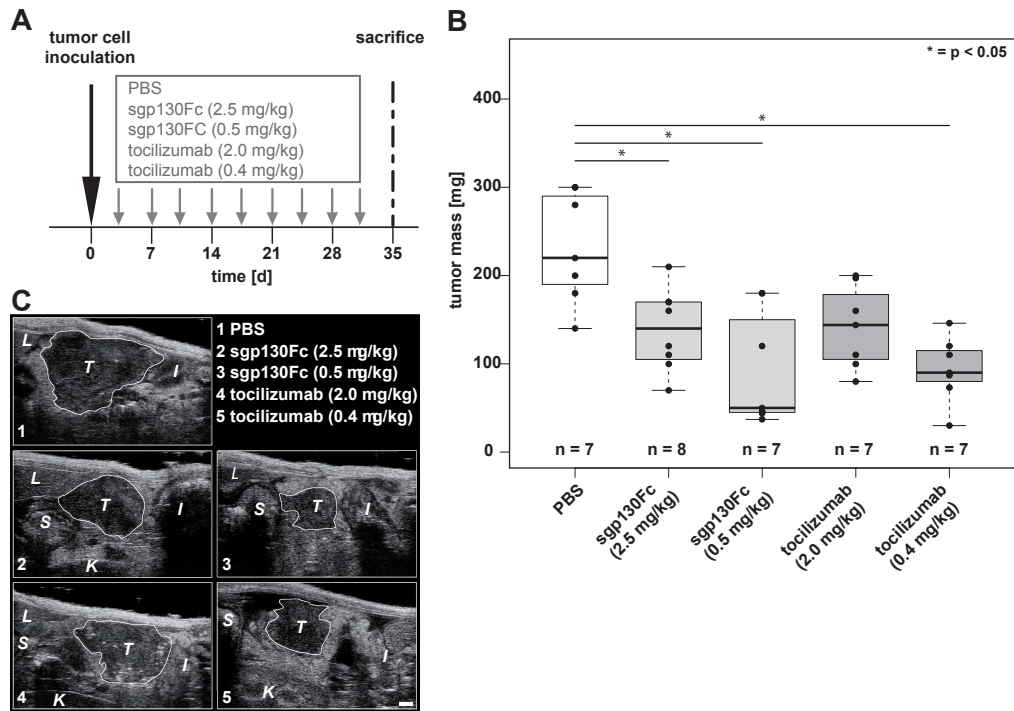


Figure 3.3: Tumor weight of orthotopic pancreatic tumors in SCID/bg mice was significantly reduced after conservative treatment with sgp130Fc or tocilizumab. 1×10^6 Colo357 cells suspended in matrigel were injected into the pancreas of SCID/bg mice. Three days after inoculation, mice were treated with a low (0.5 mg/kg) or a high dose (2.5 mg/kg) of sgp130Fc, with tocilizumab in equimolar doses (0.4 mg/kg or 2.0 mg/kg, respectively) or with PBS. The inhibitors were injected i.p. twice a week. Mice were sacrificed 35 days after tumor cell injection, and tumors were removed and weighed. B, Box plots of tumor weights observed under the different treatments. Significance was tested using a Kruskal-Wallis rank sum test followed by a Wilcoxon rank sum test with multiple testing correction by the *p*-adjustment method of Benjamini and Hochberg. C, Representative sonography images of tumors (T) in treated mice on day 34 after tumor cell inoculation. I, intestine; K, kidney; L, liver, S, stomach; scale bar = 1 mm.

3.3.4 Conservative treatment with a low dose of sgp130Fc reduces proliferation and neoangiogenesis.

The tumors were further analyzed by immunohistochemistry to better understand their pathophysiology. Interestingly, the percentage of cells expressing the proliferation marker Ki67 was significantly increased in the treated groups compared to the control group (Fig. 3.4 A). This was unexpected, as the tumors were smaller. Only tumors treated with sgp130Fc at a low concentration showed a slight decrease in the number of Ki67+ cells compared to controls (Fig. 3.4 A). We hypothesized that the tumors might reduce their proliferation at a certain size due to lack of oxygen and nutrients supply. Indeed, a correlation of tumor mass and the number of Ki67+ cells revealed a tendency of the tumors to show less Ki67+ cells with larger size. However, the group treated with a low concentration of sgp130Fc behaved differently, showing a low number of Ki67+ cells even in small tumors (Fig. 3.4 B).

Therefore, we analyzed the degree of neoangiogenesis in the tumors by CD31 immunostaining. The treatments with low concentrations of sgp130Fc or tocilizumab were evaluated, as they showed the most pronounced decrease in tumor size. Interestingly, only the tumors of the sgp130Fc-treated group showed a significantly decreased microvessel density, while the tumors of the tocilizumab treated mice showed no change (Fig. 3.4 C).

3.3.5 Adjuvant treatment of orthotopic pancreatic xenografts with sgp130Fc or tocilizumab decreases tumor recurrence and metastasis.

The adjuvant treatment model described by our group reflects the situation of those 10-20 % of all PDAC patients in whom the tumor is surgically resected [26]. In our model, the tumors were removed ten days after tumor cell inoculation, and mice were treated with the different inhibitors or PBS at the intervals indicated in Fig. 3.5 A. Tumor growth was monitored by ultrasound. Representative images on day 32 after resection are shown in Fig. 3.5 B. Finally, mice were sacrificed and analyzed for tumor recurrence, weight of recurrent tumors and number of metastases. Tumor recurrence was reduced from 7/8 mice in the control group to 5/8 in the tocilizumab-treated group and 4/8 in the sgp130Fc-treated group (Fig. 3.5 C). Not only the frequency of recurrent tumors was diminished after treatment, but also their weight, especially in the animals treated with sgp130Fc (Fig. 3.5 D). Two of the mice treated with tocilizumab showed no signs of recurrent local disease or distant metastases, while distant metastases were found in the other mice without local recurrent tumors (Fig. 3.5 C). The frequency of metastases (independent of tumor recurrence) was also influenced. Half of the tocilizumab-treated mice remained free of distant macroscopic metastases, while this was true only for 1/8 of the mice in the control- and sgp130Fc-treated groups. Furthermore, the number of mice with more than two distinct metastases was reduced in both treated

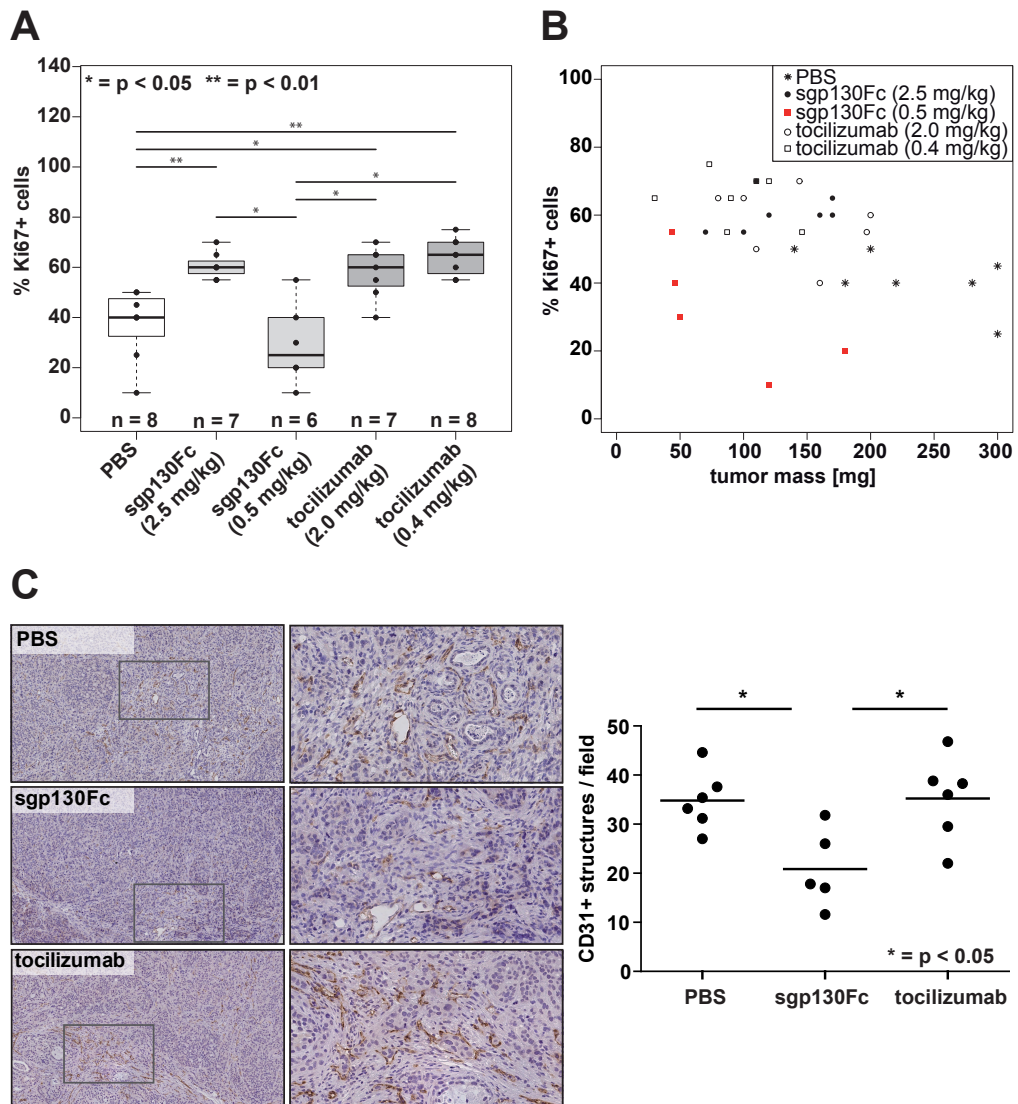


Figure 3.4: Conservative treatment with a low dose of sgp130Fc decreased the proportion of Ki67+ cells and neoangiogenesis in tumors derived from Colo357 cells. Formalin-fixed and paraffin-embedded tumors were sectioned and stained with antibodies against Ki67 or CD31. A, Box plots of the number of Ki67+ cells per 100 tumor cells. B, Correlation between tumor weight and the degree of proliferation. C, For CD31 evaluation, sections were scanned and images screened at a 10-fold magnification for vascular hotspots (left panels). These regions were examined at a 30-fold magnification (right panels) and all clearly separate CD31+ structures were counted. Representative images are shown. Three to five hotspots per tumor were analyzed in this manner and the mean value is represented by a single dot (stripchart). At least five tumors were examined in each treatment group. *P* values were calculated with Tukeys multiple comparison test following an ANOVA test.

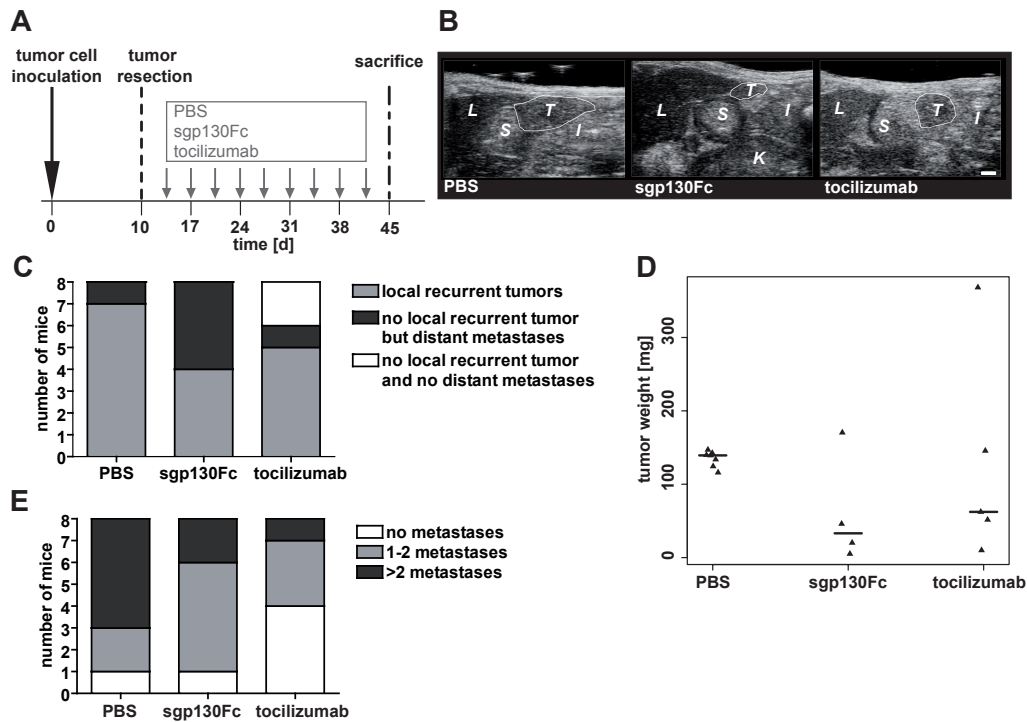


Figure 3.5: Growth of recurrent tumors or metastases was reduced after adjuvant therapy with sgp130Fc or tocilizumab. A, 1×10^6 Colo357 cells suspended in matrigel were injected into the pancreas of SCID/bg mice. Ten days after tumor cell inoculation, the tumors were resected. Treatment of the mice started three days after resection. PBS, sgp130Fc (0.5 mg/kg) or tocilizumab (0.4 mg/kg) were injected i.p. twice a week. Animals were sacrificed on the 35th postoperative day, the recurrent tumors were weighed, and all macroscopic metastases were counted. B, Representative ultrasound images on day 32 after resection. I, intestine; K, kidney; L, liver; S, stomach, T, tumor, scale bar = 1 mm. C, Proportion of mice with recurrent tumors and/or distant metastases. D, Tumor weight of recurrent tumors (line = median). E, Proportion of mice with different amounts of distant metastases.

groups compared to the control group (Fig. 3.5 E). This suggests that tocilizumab may have a stronger inhibitory effect on metastasis than sgp130Fc in this model.

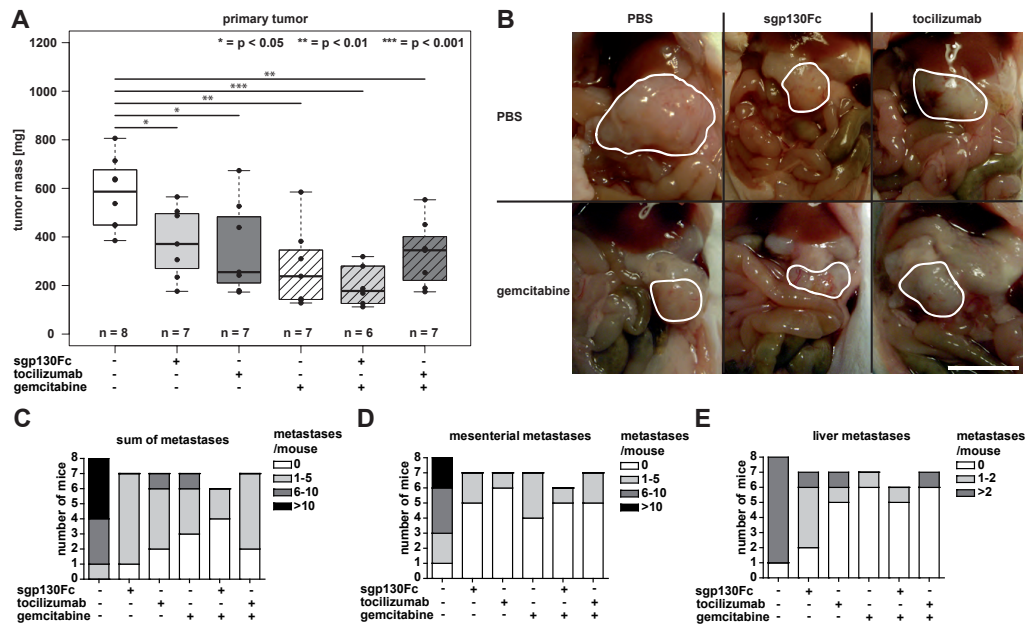


Figure 3.6: Conservative treatment with sgp130Fc and tocilizumab significantly reduced orthotopic tumor growth of PancTuI cells. 1×10^6 PancTuI cells suspended in matrigel were injected into the pancreas of SCID/bg mice. Three days after inoculation, mice were treated either with PBS, with sgp130Fc (0.5 mg/kg), with tocilizumab (0.4 mg/kg), with gemcitabine (1 mg/kg) or with a combination of gemcitabine with the IL-6 inhibitors (i.p. twice a week). Mice were sacrificed 28 days after tumor cell injection, and tumors were removed and weighed. A, Box plots of tumor weights observed under the different treatments. Significance was tested using an ANOVA test followed by pairwise t-tests with multiple testing correction by the p-adjustment method of Benjamini and Hochberg. B, Representative macroscopic images of tumors in the abdominal cavity (tumors are encircled, scale bar = 1 cm). C-E, Proportion of mice with different amounts of distant macroscopic metastases in all organs (C), in the mesenterium (D) or in the liver (E).

3.3.6 Conservative treatment with sgp130Fc or tocilizumab significantly decreases primary tumor growth of PancTuI cells *in vivo*.

To extend our analysis to other cell systems, we used the well established PancTuI model [33]. Gemcitabine was used for comparison as standard way of care and additionally in combination with both IL-6 inhibitors. Both sgp130Fc and tocilizumab significantly reduced the primary tumor mass also in this aggressive and poorly differentiated cell system (Fig. 3.6 A+B). Although gemcitabine led to a higher reduction in the mean tumor weight than sgp130Fc or tocilizumab, this difference between gemcitabine and the IL-6 inhibitors was not statistically significant (Fig. 3.6 A+B). A combination treatment with gemcitabine and sgp130Fc

showed a trend towards further improvement, but this did not reach statistical significance (Fig. 3.6 A). All treatments reduced the number of macroscopic metastases. Whereas in the untreated group 7 of 8 mice had more than six macroscopic metastases in other organs, such metastases were found only in one mouse each in the gemcitabine and tocilizumab group and in none of the mice in the other treatment groups (Fig. 3.6 C). The treatment effects were especially obvious for mesenteric metastasis: 5/8 of the PBS-treated mice had more than six macroscopic metastases while no mice in the treatment groups showed such high numbers. In this aspect, the effects of sgp130Fc and tocilizumab were comparable to that of gemcitabine (Fig. 3.6 D). Both inhibitors also reduced the number of liver metastases. Here, tocilizumab performed better than sgp130Fc, and gemcitabine as well as the combination treatments had the best effect (Fig. 3.6 E).

Additionally, we could show that the primary tumor growth of the highly aggressive murine cell line Panc02 was significantly reduced in Scid/bg mice after treatment with sgp130Fc (data not shown). In summary, sgp130Fc and tocilizumab show significant therapeutic efficacy against different aggressive human and murine pancreatic cancer cell lines in these *in vivo* models. In particular, a potential combination treatment of gemcitabine and sgp130Fc warrants further investigation regarding timing and dose-dependencies.

3.4 Discussion

In the present study, we demonstrate for the first time that the growth of human PDAC cells is significantly reduced by pharmacological inhibition of IL-6 signaling *in vivo*. The humanized monoclonal antibody tocilizumab, which completely blocks IL-6 signaling by binding to the IL-6R, is already approved for the treatment of rheumatoid arthritis, juvenile idiopathic arthritis and Castleman's disease [11]. Tocilizumab has also been successfully tested in a pre-clinical model of human oral squamous cell carcinoma [34]. However, no data examining the effect of tocilizumab on human PDAC cells are available to date. The same holds true for sgp130Fc, which specifically inhibits IL-6 trans-signaling and is currently in clinical development for inflammatory bowel diseases.

Several studies have proposed IL-6 as a potential target for the therapy of PDAC [13, 14]. Some groups already analyzed the impact of IL-6 on PDAC development *in vivo* [15, 16, 17]. However, all these studies used a mouse model of Kras-driven disease. This model allows the observation of the progression from precursor lesions (PanINs) to PDAC, as it is assumed to usually take place in human disease. These studies therefore reflect mainly the onset and early stages of pancreatic cancer. In contrast, we used an orthotopic xenograft model of human PDAC. The inoculation of an aggressive human PDAC cell line into the pancreata of mice rather reflects an advanced stage of the disease. Here, both IL-6 inhibitors led to a re-

markable reduction of tumor mass. Interestingly, sgp130Fc was slightly more effective than tocilizumab, even though tocilizumab blocks classic as well as trans-signaling. This implies that trans-signaling rather than classic signaling is the driving force of human PDAC, as it was also suggested by Lesina *et al.* in their mouse studies [15].

An orthotopic tumor is a complex network of human and murine cells. Lesina *et al.* stated that myeloid cells were the main source of IL-6 in PDAC [15]. Zhang *et al.* contradicted this finding and described IL-6 expression in several cell types within the cancer microenvironment, including pancreatic cancer cells [17]. This is in accordance with our data showing that several human PDAC cell lines express IL-6 as well as the IL-6R. Other studies also described IL-6 expression of human PDAC cells [35, 36]. Furthermore, it appears unlikely in our model that murine IL-6 produced by macrophages mediates the main tumor-promoting effect, because murine IL-6 can only bind to murine IL-6R, which is not inhibited by tocilizumab. In this scenario, tocilizumab should have no therapeutic effect. However, in our study, both inhibitors significantly reduced tumor growth. sgp130Fc showed an impact on the cellular proliferation rate and neoangiogenesis, which was not observed with tocilizumab. This difference between the inhibitors might be explained by the species difference, as neoangiogenesis might be mediated mainly by the IL-6R shed from murine stromal cells. Future studies are in progress and will investigate this aspect.

The clinical situation of approximately 10-20 % of all PDAC patients, in whom the tumor is resectable, is simulated by our adjuvant treatment scheme. Again, both inhibitors exhibited striking effects in this setting. Local tumor recurrence rate decreased from 87.5 % in the control group to 50 % in the sgp130Fc-treated and 62.5 % in the tocilizumab-treated group. Furthermore, the tumor mass of the local recurrent tumors was remarkably decreased with both substances. sgp130Fc performed again slightly better in terms of recurrence rate and tumor weight. It also led to a reduced proportion of mice with more than two macroscopic metastases. However, tocilizumab additionally led to an increase in the number of mice that showed no macroscopic metastases at all. Twenty-five percent of the mice receiving tocilizumab even remained tumor-free without any signs of local recurrent tumors or distant metastases. It is tempting to speculate that classic, cell-autonomous IL-6-signaling might become more important once the cancer cells leave their primary microenvironment and enter circulation or metastasize to distant organs. However, the exact mechanism behind this observation remains to be elucidated.

Treatment of pancreatic cancer patients remains a challenge and gemcitabine is still the standard of care for advanced PDAC without major survival benefit [37]. However, many PDAC patients show a gemcitabine resistance [38, 39]. Therefore, the search for alternative and second line therapy options is inevitable. Mitsunaga *et al.* recently suggested inhibition

of the IL-6 pathway as a candidate target for novel therapies. They demonstrated the serum level of IL-6 to be predictive of both the efficacy of gemcitabine and the prognosis of patients with advanced PDAC [40]. In our study, the treatment effect of sgp130Fc and tocilizumab was comparable to gemcitabine in PancTuI cells. Combination therapy did not lead to a statistically significant sensitisation for gemcitabine treatment. This might be due to the fact that PancTuI cells show a very gemcitabine-sensitive phenotype *in vivo*, although they are not very sensitive to gemcitabine treatment *in vitro* [38, 41]. Therefore, IL-6 trans-signaling inhibition probably may reveal only minor additional effects to gemcitabine in this model. However, this might be different in cells that are gemcitabine-resistant also *in vivo*. To further investigate the question whether combination of gemcitabine treatment and IL-6 (classic or trans-signaling) inhibition might be useful, a more detailed analysis of different treatment regimens (IL-6 inhibition prior to gemcitabine treatment, different concentrations and time points) in different cell systems would be necessary.

As human PDAC cells express all necessary IL-6 signaling molecules (IL-6, IL-6R and gp130), cell-autonomous autocrine stimulation could be one explanation for our *in vivo* findings. However, *in vitro* treatment with sgp130Fc or tocilizumab or stimulation with exogenous IL-6 or Hyper-IL-6 did not show any impact on the proliferation of Colo357 cells. This is in accordance with published data that described minor changes in the proliferation of some PDAC cells after IL-6 stimulation, while other cell lines did not show any change [42, 43]. Therefore, the inhibitors may rather influence a complex interaction between different cells of the microenvironment *in vivo*. Moreover, Bao *et al.* showed that hypoxia upregulates IL-6 and induces a more aggressive phenotype in human pancreatic cancer cells [44]. As PDAC is a hypoxic tumor [45], low oxygen supply might contribute to the differences between our *in vitro* and *in vivo* findings and highlights the importance of animal experiments in cancer research.

It is well established that IL-6 levels are elevated in the serum of PDAC patients and correlate with a worse prognosis [13]. Our findings strongly support the use of IL-6 inhibitors as a new treatment option for PDAC. Jamieson *et al.* showed that the survival of PDAC patients after resection is worse when the patients exhibit higher systemic inflammation [46]. IL-6 inhibition might thus be an option to reduce this systemic inflammation, positively influencing the quality of life of cancer patients after resection in addition to reducing tumor recurrence and metastasis.

Moreover, a case report recently described the successful use of tocilizumab in treating cancer cachexia [47]. IL-6 expression has been shown to be significantly higher in pancreatic tissue of patients with cachexia compared to noncachexic patients [48]. As many PDAC patients suffer from cachexia [49], IL-6 blockade may not only be beneficial in terms of tumor

progression, but also for stabilizing the overall status of the patients. However, treatment of cancer patients with tocilizumab is controversial, because it is immunosuppressive and might increase the risk of infections [50]. The selective trans-signaling inhibitor sgp130Fc might elegantly solve this conflict, as it does not favor opportunistic infections [11, 12] and was effective in reducing tumor growth in this study.

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Conflict of interest

S.R.-J. is an inventor on gp130 patents owned by CONARIS and a shareholder of the CONARIS Research Institute AG (Kiel, Germany), which is commercially developing sgp130Fc as a therapy for inflammatory diseases. G.H.W. is employed by the CONARIS Research Institute AG and an inventor on gp130 patents owned by CONARIS. All other authors declare no competing financial interests.

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3.5 Additional data and further perspectives

In this section, I show additional data that were not published in the original article but that broaden our understanding about IL-6 signaling in pancreatic cancer.

3.5.1 Material and Methods

Quantitative real-time polymerase chain reaction (qPCR)

RNA was isolated using the peqGOLD Total RNA Kit (Peqlab, Erlangen, Germany). RNA concentration was measured in a Nanodrop spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany) and quality-checked on a 1 % agarose gel. 2 µg of RNA were reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and diluted 100-fold in nuclease-free water. 2 µl of diluted cDNA were used in a 20 µl reaction with FastSybr Green mastermix (Applied Biosystems/Life Technologies). The primer sequences are depicted in table 3.2, and the following conditions were used: initial denaturation: 95 °C, 20 sec; denaturation: 95 °C, 3 sec; annealing/extension: 60 °C, 30 sec (usually 40 cycles). Specificity of the product was verified by melt curve analysis and agarose gel electrophoresis.

name	sequence 5'-3'	amplicon [bp] (mRNA/genomic)	reference
hu_CEA_For	CTTTATCGCCAAAATCACGC	138/6195	http://primerdepot.nci.nih.gov
hu_CEA_Rev	CCAGCTGAGAGACCAGGAGA	138/6195	http://primerdepot.nci.nih.gov
hu_CEACAM6_For1	GCATGTCCCCTGGAAGGA	179/1076	[1]
hu_CEACAM6_Rev1	CGCCTTTGTACCAGCTGTAA	179/1076	[1]
hu_RPL22_For	TCGCTCACCTCCCTTTCTAA	250/6652	[2]
hu_RPL22_Rev	TCACGGTGATCTTGCTCTTG	250/6652	[2]
hu_RPL27_For	ATCGCCAAGAGATCAAAGATAA	123/2693	[2]
hu_RPL27_Rev	TCTGAAGACATCCTTATTGACG	123/2693	[2]

Table 3.2: Primer sequences used for qPCR

Preparation of cell lysates and western blots

Cell lysates and western blots were performed as described earlier in this chapter (section 3.2.9). Some blots were incubated with horse radish peroxidase-coupled secondary antibodies (#7074/7076 Cell Signaling Technology/New England Biolabs, Schwalbach, Germany) and a substrate for electrochemiluminescence (ECL) (GE Healthcare, Munich, Germany) instead of fluorophor-coupled antibodies. Films (Amersham Hyperfilm ECL, GE Healthcare) were used to detect the signal. Antibodies are shown in table 3.3.

antigen	product number	supplier
CEACAM5	T84.66	kind gift of Prof. Neumaier, Mannheim
CEACAM6	AM02001PU-N	Acris Antibodies GmbH, Herford, Germany
P-ERK1/2	#9106	Cell Signaling Technology/New England Biolabs, Schwalbach, Germany
ERK1/2	#9102	Cell Signaling Technology/New England Biolabs, Schwalbach, Germany
P-p38	#9211	Cell Signaling Technology/New England Biolabs, Schwalbach, Germany
p38	#9217	Cell Signaling Technology/New England Biolabs, Schwalbach, Germany
bcl-2	#2872	Cell Signaling Technology/New England Biolabs, Schwalbach, Germany
bcl-xL	51-6646GR	BD Pharmingen, Franklin Lakes, NJ, USA
c-myc	#5605	Cell Signaling Technology/New England Biolabs, Schwalbach, Germany
p21	#2946	Cell Signaling Technology/New England Biolabs, Schwalbach, Germany
survivin	#2802	Cell Signaling Technology/New England Biolabs, Schwalbach, Germany
E-cadherin	610181	R&D Systems, Wiesbaden, Germany
HIF-1 α	610959	BD Biosciences, Franklin Lakes, NJ, USA

Table 3.3: Antibodies used for western blotting

Stimulation of Colo357 cells

To prepare conditioned medium, 1×10^6 Colo357 cells were seeded in a T75 flask. After 48 h the medium was changed. After another 48 h, the medium was collected and centrifuged for 5 min at 1400 rpm to remove cells. The supernatant was collected, transferred to a new tube and centrifuged again for 15 min at 5000 rpm to remove debris. The supernatant was aliquoted and stored at -20°C until use.

To analyze the impact of conditioned medium, Colo357 cells were seeded in 6-well plates. After 48 h, the medium was changed to serum-free medium. The next day, cells were pre-incubated with the inhibitors for 1 h and subsequently stimulated either with IL-6 or with conditioned medium for 15 min. Afterwards, the cells were lysed as previously described in section 3.2.9 and analysed by western blots.

To analyze the CEACAM expression, Colo357 cells were seeded in 6-well plates. After 48 h, the medium was changed to serum-free or normal medium. The next morning, cells were stimulated with IL-6 in serum-free medium or with tocilizumab in normal medium. After 24 h, RNA was isolated and qPCR performed as described in section 3.5.1.

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections were prepared as previously described in this chapter (section 3.2.6). CEACAM5 and cleaved caspase-3 (#9664, Cell Signaling) staining was performed using the autostainer Bond™ Max System (Leica-Menarini, Berlin, Germany). For P-STAT3 staining, antigen retrieval was done in citrate buffer (pH 6) using the Pascal Pressure Cooker (Dako). All washing steps were done at room temperature (RT) with tris-buffered saline (TBS). Endogenous peroxidase activity was blocked with Lab Vision Hydrogen Peroxide Block (Thermo Fisher Scientific) for 15 min at RT. Unspecific binding sites were blocked with Lab Vision Ultra V Block for 5 min at RT (Thermo Fisher Scientific). Slides were incubated with primary antibody overnight at 4 °C (#9145 Cell Signaling; dilution in Antibody Diluent, Zytomed, Bargteheide, Germany). A universal immunoperoxidase polymer (Histofine Simple Stain MAX PO, Nichirei Biosciences, Tokyo, Japan) was added for 30 min at RT. Sections were stained with diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA) for 10 min and counterstained with hemalum. Slides were analyzed and quantified by a qualified pathologist who was not involved in the study design (A. Gontarewicz).

Clonogenic growth

Colo357 cells were diluted to a concentration of 3.33 cells/ml and distributed to four falcon tubes. One falcon tube only contained medium, in one was IL-6 (final concentration of 100 ng/ml), in one sgp130Fc [10 µg/ml] and in the last tocilizumab [8 µg/ml]. The cell suspensions were distributed on three 96-well plates per treatment. 100 µl were pipetted into each well, meaning that statistically one cell was seeded in every third well of a 96-well plate. The outer wells were excluded and just filled with medium, because they often show a higher evaporation of medium. After two weeks, 10 µl alamarBlue® solution (Invitrogen/Life Technologies) were added to the wells. Twenty-four hours later, the emission at 595 nm was measured in the SPECTRA Fluor Plus fluorescence reader (Tecan, Maennedorf, Switzerland) with excitation at 550 nm (manual gain: 100). Values above 15000 were regarded as positive. This was validated by microscopy, showing cell clones in these wells while the others were cell-free. Positive wells were counted per plate.

Orthotopic inoculation of Panc02 cells

The procedure for the inoculation of Panc02 cells into Scid/bg mice was almost the same as for Colo357 cells. However, cells were detached with trypsin-EDTA instead of accutase and only 5×10^4 cells were inoculated. Treatment with sgp130Fc [0.5 mg/kg bodyweight] started three days later and continued twice a week. Mice were sacrificed on day 14. The surgical procedure remained the same for sgp130Fc-transgenic and C57/BL6 mice. Five-week old

female sgp130Fc-transgenic mice were kindly provided by the group of Stefan Rose-John. Age-matched C57/BL6 mice were obtained from Charles River (Sulzfeld, Germany) and housed in the same facility. Panc02 cells were inoculated into the pancreas as previously described. Mice were routinely examined for any signs of physical distraction and tumor growth monitored by ultrasound. On day 14, the mice were sacrificed.

3.5.2 Results and Discussion

Conservative treatment with sgp130Fc reduces the STAT3 phosphorylation of stromal cells

To better understand the effect of IL-6 inhibition in the conservative treatment scheme, we stained the tumor tissue from Colo357-derived tumors for phosphorylated STAT3 (P-STAT3). P-STAT3 was observed in tumor cells as well as in cells of the surrounding tumor stroma (Fig. 3.7 A). Therefore, we separately analyzed the amount of P-STAT3-positive cells for the tumor and stromal compartment. In the tumor compartment, the amount of P-STAT3+ cells largely varied between individual tumors but there was no statistical difference between the treatment groups (Fig. 3.7 B). Interestingly, significantly less stromal cells were P-STAT3+ after treatment with sgp130Fc (Fig. 3.7 C). As sgp130Fc also inhibits trans-signaling via the murine IL-6R while tocilizumab only targets the human receptor, this suggests a role of the murine IL-6R for the P-STAT3 of the stromal compartment. This finding might also explain the reduced amount of CD31+ cells in sgp130Fc-treated mice (compare section 3.3.4). The finding, that P-STAT3 was not reduced in the tumor cells was surprising at the first sight, because tocilizumab as well as sgp130Fc inhibit IL-6 signaling and therefore probably also the STAT3 phosphorylation. Yet, IL-6 is not the only molecule that induces a STAT3 phosphorylation and in tumor cells, other factors may start to activate STAT3 when IL-6 is absent. *In vitro*, IL-6 inhibition also did not lead to a complete P-STAT3 inhibition in tumor cells (see next section).

Colo357 cells show an endogenous STAT3 phosphorylation, which is only partly inhibited by tocilizumab

To further analyze the phosphorylation of STAT3 in pancreatic tumor cells, we analyzed the endogenous levels of P-STAT3 in Colo357 cells at different time points. In serum-containing medium, an endogenous P-STAT3 was observed after 24 h, which increased over time (Fig. 3.8 A). Interestingly, after 24 h in serum-free medium, the endogenous STAT3 phosphorylation almost disappeared but reappeared after 48 and 72 h (Fig. 3.8 A). These observations argue against a continuous intrinsic phosphorylation of STAT3 and rather suggest that the activation is mediated by secreted factors, which accumulate over time in the medium. To prove this hypothesis, we serum-starved Colo357 cells overnight to reduce the endogenous

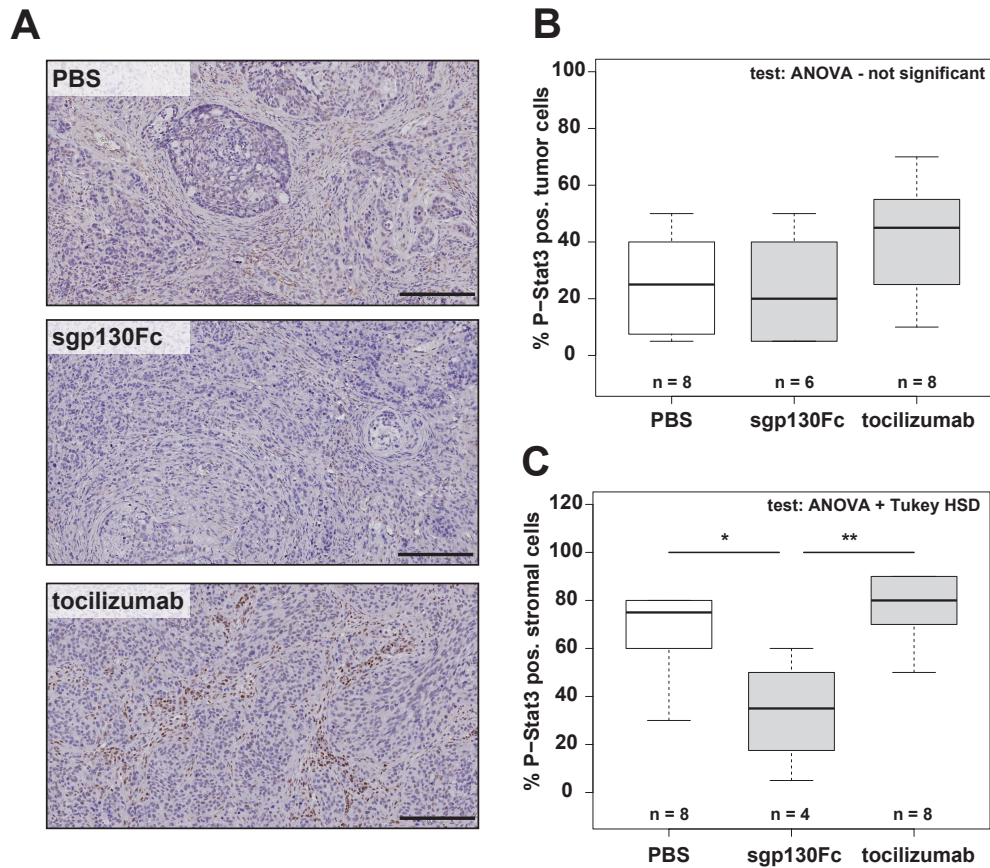


Figure 3.7: Conservative treatment with sgp130Fc leads to a reduction of P-STAT3+ stromal cells. Colo357 cells were orthotopically injected into the pancreas of Scid/bg mice and treated with IL-6 inhibitors. After 35 days, the tumors were removed, fixed in formalin and embedded in paraffin and sectioned. Sections were stained for P-STAT3 (**A**, representative pictures) and analyzed for P-STAT3+ tumor cells (**B**) or stromal cells (**C**). scale bar = 200 μ m

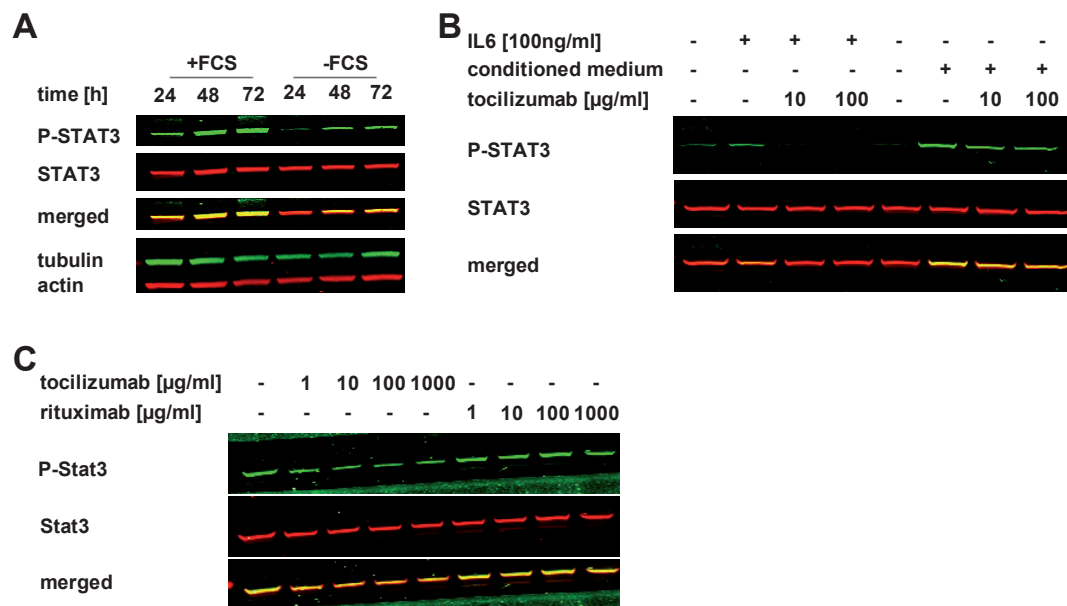


Figure 3.8: The endogenous STAT3 phosphorylation of Colo357 cells is only slightly reduced by tocilizumab. **A**, Colo357 cells were cultured in normal (+FCS) or serum-free (-FCS) medium for different time points. Cell lysates were analyzed for STAT3 phosphorylation in western blot. To ensure equal protein content, the protein levels of β -actin and tubulin were analyzed. **B**, Cells were serum-starved overnight and pre-incubated with tocilizumab for 1 h. Subsequently, cells were treated with IL-6 [100 ng/ml] or conditioned medium from Colo357 cells for 15 min. Cell lysates were analyzed for STAT3 phosphorylation in western blot. **C**, Colo357 cells were seeded and subsequently incubated with different concentrations of tocilizumab or rituximab (as negative control) for 48 h. Cell lysates were analyzed for STAT3 phosphorylation in western blot.

P-STAT3 and subsequently incubated them with conditioned medium from Colo357 cells for only 15 min. In comparison to cells treated with serum-free medium alone, the cells stimulated with conditioned medium clearly showed an increased P-STAT3. The level was similar to cells incubated with IL-6 (Fig. 3.8 B). As IL-6 is produced and secreted by Colo357 cells (compare chapter 3.3.1) and IL-6 is known to activate STAT3, we analyzed whether a blockade of IL-6 would inhibit this STAT3 phosphorylation. While treatment with tocilizumab almost completely inhibited the IL-6-induced STAT3 phosphorylation, it only slightly reduced the phosphorylation of STAT3 induced by conditioned medium (Fig. 3.8 B). Furthermore, incubation of Colo357 cells with tocilizumab for 48 h only marginally reduced the amount of P-STAT3 (Fig. 3.8 C). These observations suggest IL-6 to be only one factor that accounts for the phosphorylation of STAT3 in Colo357 cells. Additional factors are likely to be involved. This is in line with the *in vivo* findings that IL-6 inhibition does not completely inhibit the STAT3 phosphorylation in tumor cells (see chapter 3.5.2). Additional factors known to activate STAT3 are for example other cytokines like leukemia inhibitory factor (LIF), IL-5, IL-9, IL-10, IL-11, IL-12, IL-21, IL-22, IL-27, IFN- γ , TNF- α , macrophage inflammatory protein-1 α (MIP-1 α), and growth factors like EGF, TGF α , M-CSF and GM-CSF [3]. Further studies are needed to identify, which STAT3-activating factors are secreted by Colo357 and are present in tumor tissues.

Cleaved caspase-3 is slightly increased in tumors treated with tocilizumab

To test whether apoptosis was induced by the conservative treatment of tumors, we stained the tumor tissues for cleaved caspase-3. While most slides of the control- and sgp130Fc-treated tumors were negative, the tocilizumab-treated tumors showed some caspase-3-positive cells. Yet, only 5-10 % of the tumor cells were positive, and morphological analysis of apoptotic bodies in hematoxylin/eosin staining did not reflect this change between treatment groups. Again the time point of the analysis might be of relevance. It is possible that apoptosis was induced at earlier time points and the apoptotic bodies removed by macrophages or the tumor cells themselves (as PDAC cells are known to have phagocytic activity, personal communication by Prof. Kalthoff, Kiel).

Treatment of tumor-bearing mice did not influence their weight

Several studies described a relationship between IL-6 and cancer cachexia (for details see chapter 2.3.1). Therefore, we analyzed the weight of the mice in our models. In the first days after surgery, mice lost weight. This is probably due to stress and weakening after surgery. But mice recovered after approximately one week and started to regain weight. Treatment with tocilizumab or sgp130Fc did not significantly alter the weight of mice (Fig. 3.10). This is presumably different in tumor models, in which mice suffer from severe weight loss. Strassmann

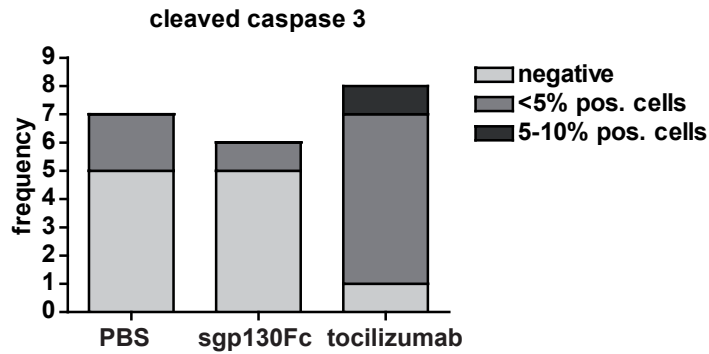


Figure 3.9: Conservative treatment with tocilizumab slightly increases the amount of cleaved caspase-3 positive tumor cells. Colo357 cells were orthotopically injected into the pancreas of Scid/bg mice. After 35 days the tumors were removed, fixed in formalin and embedded in paraffin and sectioned. Sections were stained for cleaved caspase-3, and the percentage of positive tumor cells were estimated per tumor.

et al. showed in the murine C-26 cachexia model, that treatment with an IL-6 neutralizing antibody attenuated the development of weight loss [4].

The expression of CEACAM5/6 is influenced by stimulation or inhibition of IL-6 *in vitro* but not *in vivo*

We previously observed that IL-6 trans-signaling leads to an upregulation of CEACAM5 and CEACAM6 in colorectal cancer cells (see chapter 4.3.3). Therefore, we were interested whether this was also the case for pancreatic cancer cells. As Colo357 cells were responsive to IL-6 classic signaling, we treated them with IL-6 *in vitro*. Serum-free medium was used for the stimulation, because cells responded stronger to IL-6 when they were serum-starved before. Additionally, we inhibited endogenous IL-6 as we knew that Colo357 produced IL-6 themselves. For blockade of endogenous IL-6 signaling, cells were treated with tocilizumab in normal medium, as the IL-6 production was stronger when the cells were grown in serum-containing medium (see section 3.3.1). The expression of CEACAM5 and also of CEACAM6 was increased by IL-6 and decreased by tocilizumab (Fig. 3.11A).

To analyze if this connection can also be observed *in vivo*, we stained tumor sections of tumors derived from Colo357 cells in Scid/bg mice, which had been treated with the IL-6 inhibitors tocilizumab and sgp130Fc in a conservative manner, with an anti-CEACAM5 antibody. Only few cells were CEACAM5-positive and the antibody accumulated in the necrotic areas. However, no difference was observed between the treatment groups (Fig. 3.11 B). As CEACAM5 is released and therefore might be difficult to detect in tumor sections, we also analyzed the expression on the mRNA level in the same tumors. Tocilizumab-treated tumors

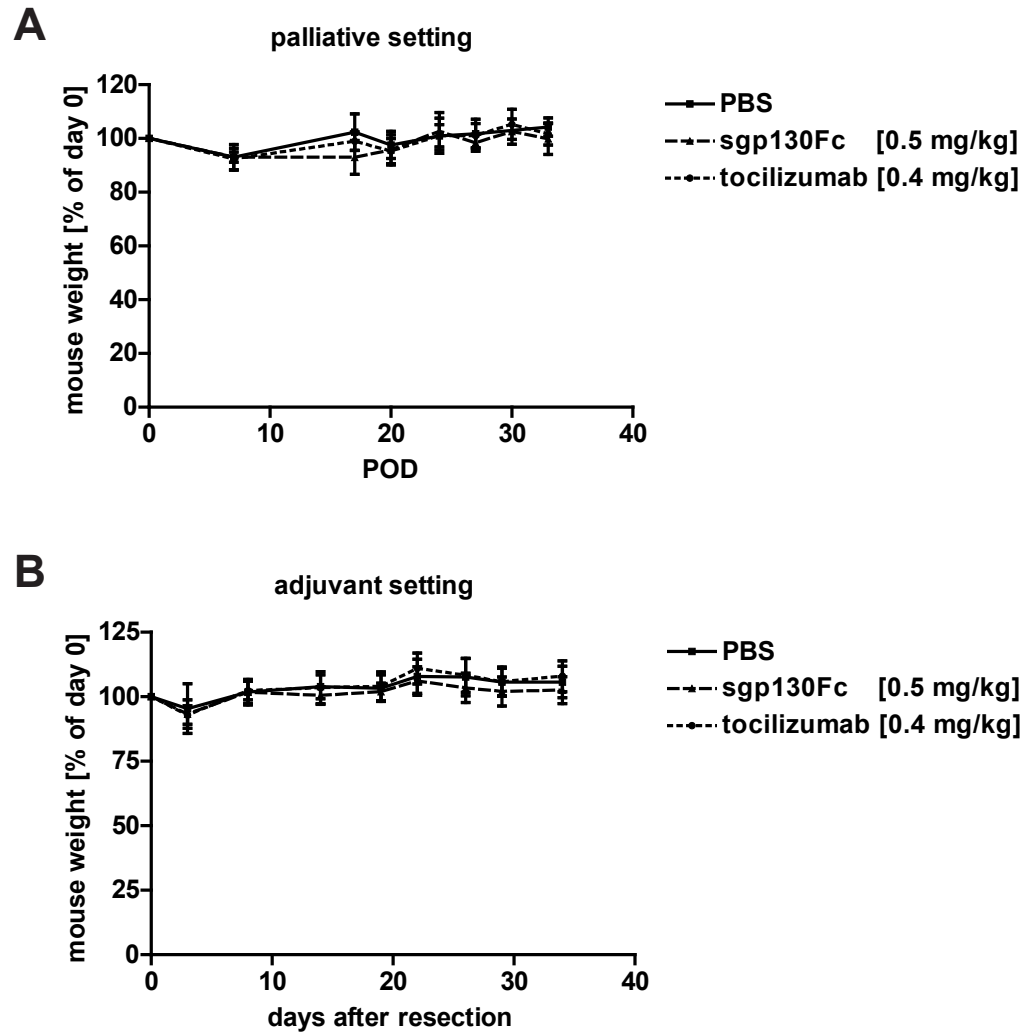


Figure 3.10: Treatment with sgp130Fc or tocilizumab does not significantly change the body-weight of tumor-bearing mice. Colo357 cells were orthotopically injected into the pancreas of Scid/bg mice. Mice were regularly weighed after surgery. In the palliative setting, the weight before surgery was considered as 100%. **POD**, postoperative day. In the adjuvant setting, the tumor was resected after ten days. The weight on the day of the resection was considered to be 100%.

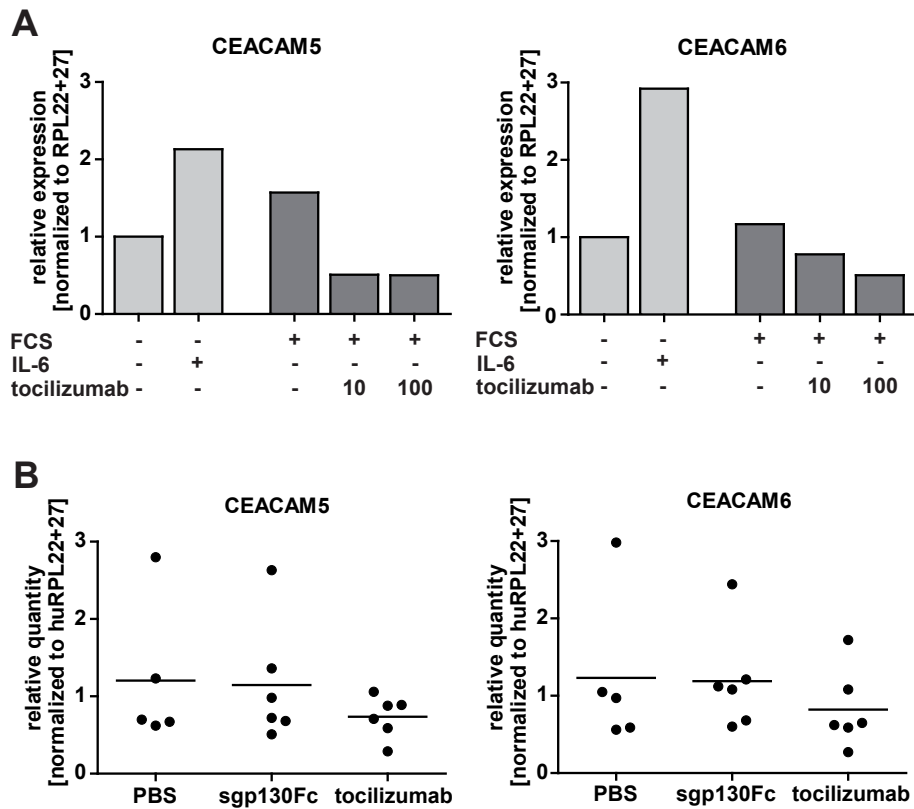


Figure 3.11: IL-6 inhibition decreases the expression of CEACAM5/6 *in vitro* but not *in vivo*. **A**, Colo357 cells were treated with IL-6 [100 ng/ml] in serum-free medium or with tocilizumab [10 or 100 µg/ml] in serum-containing medium for 24 h. RNA was isolated and qPCR performed to analyze the expression of CEACAM5/6. **B**, Colo357 cells were orthotopically injected into the pancreas of Scid/bg mice. After 35 days, the tumors were removed and either formalin-fixed and paraffin embedded (FFPE) or immediately frozen in liquid nitrogen. FFPE tumors were sectioned and stained for CEACAM5. **C**, RNA was isolated from cryo-preserved tumors and analyzed in qPCR for CEACAM5/6. Human RPL22 and RPL27 were used as reference genes. The line shows the mean of each group.

showed a trend towards decreased CEACAM5/6 expression, but this difference was not statistically significant (Fig. 3.11 B).

Again, we observed differences between *in vitro* and *in vivo* data, showing that the simple treatment of tumor cells *in vitro* does not necessarily mirror the behavior of tumor cells *in vivo*, where they are surrounded by a complex microenvironment. Theoretically, the *in vitro* finding of a CEACAM5/6 downregulation after IL-6 inhibition by tocilizumab could suggest a possible mechanism for the decreased tumor growth after IL-6 inhibition *in vivo*, as CEACAM5 and CEACAM6 were shown to contribute to epithelial-mesenchymal transition, migration, invasion and metastasis, as well as to inhibit anoikis [5, 6, 7, 8, 9, 10]. Yet, the analysis of the tumor material argues against this hypothesis and suggests a different mechanism. On the other hand, one has to keep in mind that we only analyzed the endpoint of the experiment. Therefore, we cannot completely rule out that IL-6 inhibition might have an impact on CEACAM5/6 expression in earlier stages of tumor development. It would be interesting, to analyze tumors in these early stages in further experiments. As mice do not express CEACAM5 and CEACAM6 [11], most genetically engineered mouse models of pancreatic cancer ignore the potential role of CEACAM5/6. In contrast, our xenograft model enables the analysis of these important tumor markers because the examined human pancreatic cancer cell lines express CEACAM5 and CEACAM6.

Stimulation with exogenous IL-6 or inhibition of endogenous IL-6 does not influence the clonogenic growth of Colo357 cells *in vitro*

We demonstrated that IL-6 did not influence the overall proliferation of Colo357 cells *in vitro* (see 3.3.2). Thus, we were interested whether IL-6 would play a role in the outgrowth of single cell clones and performed a clonogenic growth assay. As in the proliferation assay, treatment with IL-6 as well as inhibition with tocilizumab or sgp130Fc did not influence the mean number of single cell clones per plate (Fig. 3.12).

***In vitro* treatment with exogenous IL-6 stabilizes the expression of HIF-1 α but does not influence the expression of p21, bcl-xL, bcl-2 and survivin in Colo357 cells**

To better understand the effect of exogenous IL-6 on Colo357 cells, they were serum-starved overnight and subsequently treated with IL-6. At different time points, the cells were analyzed. As mentioned earlier, IL-6 led to a phosphorylation of STAT3 as early as 15 min after treatment. The endogenous P-STAT3 was very low at this time point due to the serum starvation. However, the endogenous P-STAT3 of the untreated cells increased over time, and at 48 h after treatment, IL-6-treated and untreated cells reached the same level of P-STAT3 (Fig. 3.13 A). IL-6 was also shown to activate ERK1/2 (p44/42 MAPK) in other cell lines [12]. The endogenous P-ERK in untreated cells was higher than P-STAT3. ERK1/2 was

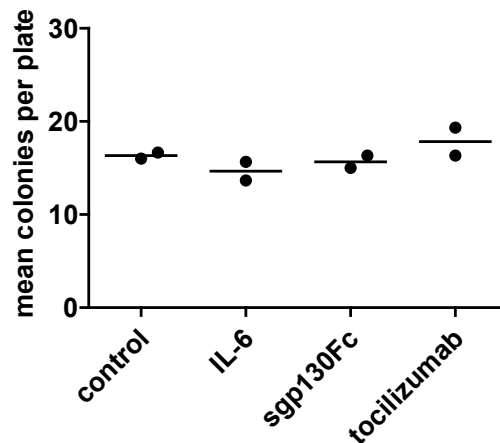


Figure 3.12: IL-6 does not influence the growth of single cell clones *in vitro*. Colo357 cells were diluted, seeded in 96-well plates (0.3 cells/well) and treated with IL-6 [100 ng/ml], sgp130Fc [10 µg/ml] or tocilizumab [8 µg/ml]. After two weeks the plates were incubated with alamarBlue® for 24 h. Positive wells were counted per plate and microscopically verified to contain cell clones. The graph shows two independent biological experiments. Each dot represents the mean of three plates in one experiment and the line the mean of both experiments.

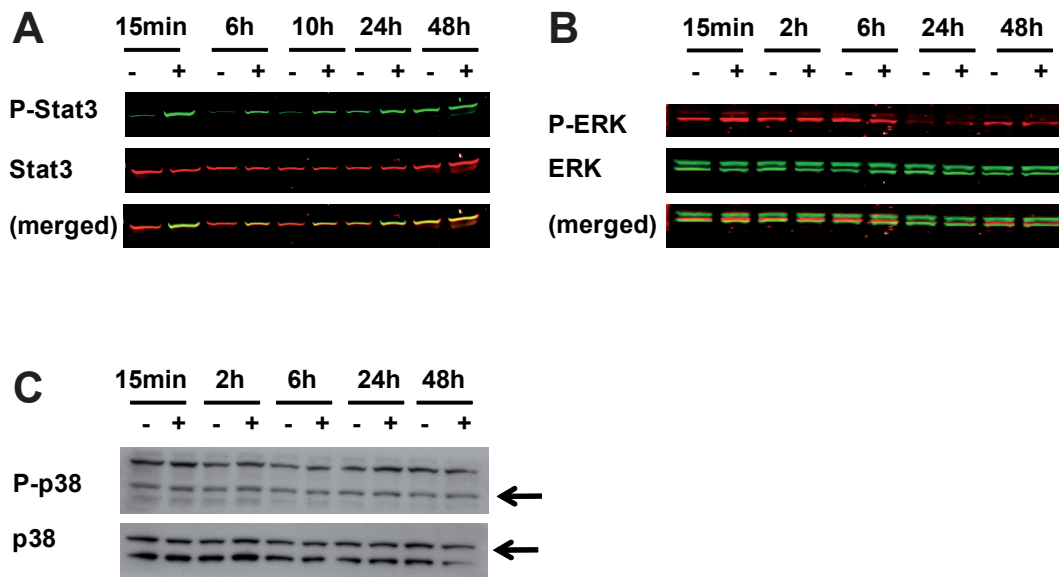


Figure 3.13: IL-6 induces the phosphorylation of STAT3 and ERK in Colo357 cells *in vitro*. Colo357 cells were seeded, serum-starved overnight and treated with IL-6 [100 ng/ml]. At the indicated time points, cells were lysed and lysates analyzed for different proteins in western blots.

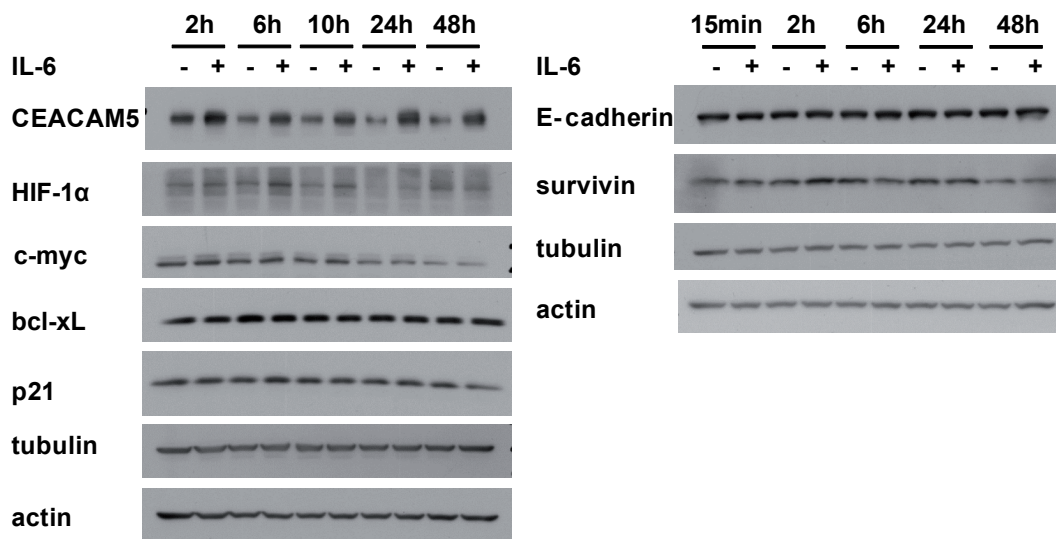


Figure 3.14: IL-6 stabilizes HIF-1 α expression in Colo357 cells. Colo357 cells were seeded, serum-starved overnight and treated with IL-6 [100 ng/ml]. At the indicated time points, cells were lysed and lysates analyzed for different proteins in western blots.

also phosphorylated after 15 min of IL-6 treatment (Fig. 3.13 B). After 2 h, no difference in the level of P-ERK1/2 could be observed anymore between IL-6-treated and untreated cells. IL-6 did not obviously alter the phosphorylation of p38 MAPK in Colo357 cells (Fig. 3.13 C).

As IL-6 was described to influence the expression of different proteins [13, 14, 15, 16, 17], we analyzed their expression by western blot analysis. None of the described proteins involved in proliferation and survival (p21, bcl-xL, survivin, c-myc) were changed in their steady-state level after treatment with IL-6 in our setting. Moreover, the expression of E-cadherin, which is involved in epithelial-mesenchymal transition (EMT), was not changed after IL-6 incubation. Yet, the expression of CEACAM5 was increased as early as 2 h after incubation with IL-6 (as described earlier in this section). Furthermore, HIF-1 α was stabilized at 6 and 10 h after treatment with IL-6. This effect was also observed in colorectal cancer cell lines and is described in more detail in chapter 4.

Inhibition of IL-6 transsignaling reduces tumor growth of Panc02 cells in immunodeficient and immunocompetent syngeneic mice

In the experiments described so far, we analyzed the growth of human tumor cells in immunodeficient mice. However, the immune system plays an important role in tumor growth. Therefore, we were interested whether inhibition of IL-6 trans-signaling also reduces tumor growth in immunocompetent mice. To do so, we needed to switch to a syngeneic model, and

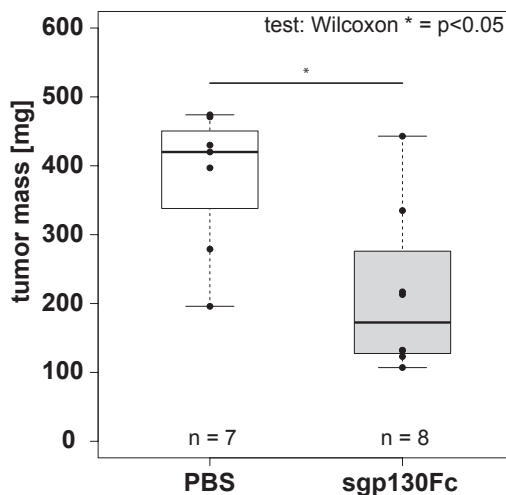


Figure 3.15: Conservative treatment with sgp130Fc significantly reduces the tumor growth of Panc02 cells in immunodeficient mice. Panc02 cells were orthotopically injected into the pancreas of SCID/bg mice. After three days, mice were treated with PBS or sgp130Fc [0.5 mg/kg bodyweight]. The mice were sacrificed after two weeks and the tumors dissected and weighed.

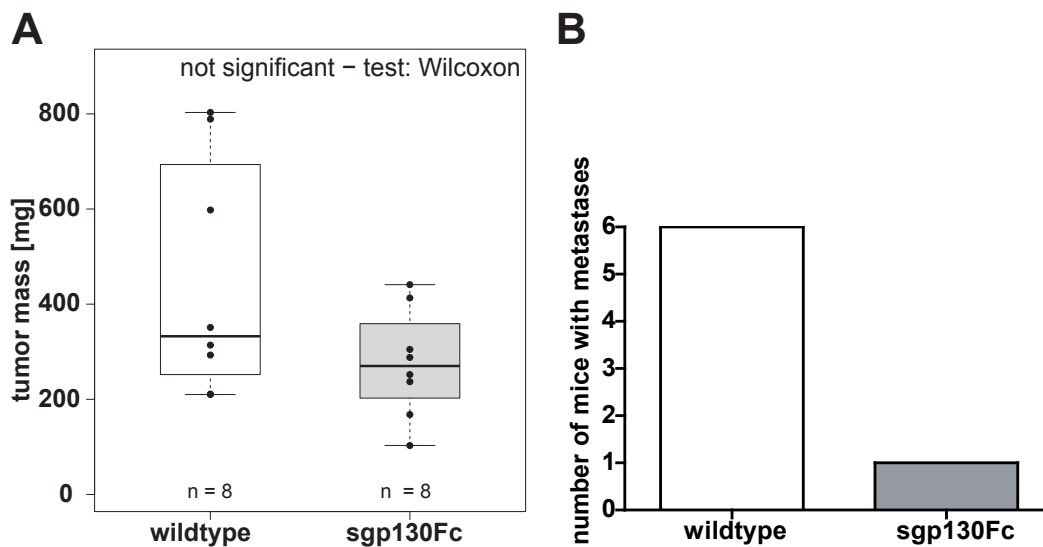


Figure 3.16: Inhibition of IL-6 transsignaling reduces the tumor growth of Panc02 cells in mice. **A**, Panc02 cells were orthotopically inoculated into the pancreas of sp130Fc-transgenic or wildtype C57/BL6 mice. After two weeks, the mice were sacrificed. The tumors were dissected and weighed (**A**) and the mice carefully examined for macroscopic metastases (**B**).

chose the cell line Panc02, which was derived from C57/BL6 mice [18]. In a first step, we inoculated them into the previously used Scid/bg mice. Treatment with sgp130Fc led to a significant reduction of primary tumor mass (Fig.3.15A).

In a second experiment, we orthotopically inoculated Panc02 cells either into C57/BL6-wildtype mice or sgp130Fc-transgenic mice. These mice produce a constant level of sgp130Fc and therefore have a better bioavailability of the inhibitor [19]. In this experiment, sgp130Fc again led to a reduction of tumor growth (Fig. 3.16 A). However, the effect did not reach statistical significance. But the amount of macroscopic metastases was clearly diminished in the sgp130Fc-transgenic mice. While 6/8 mice in the wildtype group showed macroscopic metastases (some had more than ten macroscopic metastases per mouse), only one mouse of the transgenic mice had one metastasis (Fig.3.16 B). Interestingly, we also observed reduced wound-healing in sgp130Fc-transgenic mice. These experiments were interesting as they demonstrated that inhibition of trans-Signaling is also promising in immunocompetent mice. Further experiments with less aggressive cell lines are planned, and the analysis of the tumor microenvironment in these murine models will help to better understand the role of IL-6 in tumorigenesis.

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Chapter 4

Interleukin-6 trans-signaling increases the expression of CEACAM5 and CEACAM6 in colorectal cancer cells

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Abstract

Colorectal cancer (CRC) is among the five most frequent causes for cancer-related deaths in Europe. One of the best tumor-associated antigens for CRC is carcinoembryonic antigen-related cell adhesion molecule5 (CEACAM5), which is involved in cell adhesion, migration, anoikis, tumor invasion and metastasis. Its family member CEACAM6 is also upregulated in adenomas and carcinomas of the colon and an independent predictor of poor survival. Some studies previously showed that CEACAM5 can be upregulated by the inflammatory molecule interleukin-6 (IL-6) that is important for the tumorigenesis of CRC and signals via two pathways (classic and trans-signaling). Yet, other studies could not confirm this finding and the role of trans-signaling has not been analyzed. Moreover, the impact of IL-6 on the expression of CEACAM6 has not been examined. In this study, we show a weak upregulation of CEACAM5 and CEACAM6 by classic IL-6 signaling, but a strong increase by IL-6 trans-signaling. This upregulation depends on the phosphorylation of STAT3 and may contribute to the tumor-promoting role of IL-6.

4.1 Introduction

CRC is still one of the leading causes of cancer deaths in Europe. According to predictions for the year 2014, it ranks second in men and third in women [1]. Several risk factors ex-

ist, including smoking, alcohol consumption, diabetes and inflammation [2, 3]. The link between inflammation and tumorigenesis is exemplified by patients with colitis-associated cancer (CAC). These are CRC patients that have previously suffered from inflammatory bowel disease (IBD). It is well-known that IBD patients have a higher risk of developing CRC (or CAC in this case) [4, 5].

One of the cytokines involved in IBD as well as in CRC is IL-6 [6]. IL-6 is a pleiotropic cytokine involved in various processes of innate and adaptive immunity [7, 8]. In the classic IL-6 signaling, IL-6 binds to the membrane-bound IL-6 receptor (IL-6R), which subsequently transmits the signal via the recruitment and homodimerization of two glycoprotein 130 (gp130) subunits. Thus, an intracellular cascade is activated involving signal transducer and activator of transcription-3 (STAT3), mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) activation. Whereas gp130 is ubiquitously expressed, IL-6R expression is restricted to a few cell types. However, a soluble form of IL-6R (sIL-6R) can be generated by protease-mediated receptor shedding from the membrane or by alternative splicing. In contrast to some other soluble receptors, the sIL-6R does not act as an antagonist. Instead, it binds to its ligand and trans-activates cells that only express gp130. This process was termed trans-signaling. It is selectively inhibited by a naturally occurring soluble form of gp130 (sgp130). This knowledge was used to generate a potent and selective inhibitor of trans-signaling by fusing the sgp130 protein to the Fc part of a human IgG antibody. It is called sgp130Fc or FE 999301 and is already in clinical development for IBD.

Several studies proved a significant role of IL-6 in IBD as well as in CRC. They were recently reviewed by Waldner and Neurath, who even called IL-6 the "master regulator of intestinal disease" [6]. Interestingly, in most studies the pro-inflammatory and tumor-promoting activity of IL-6 was mediated via IL-6 trans-signaling [6, 9].

The serum-level of IL-6 was significantly associated with high serum-levels of CEACAM5 [10, 11]. CEACAM5 (also called carcinoembryonic antigen, CEA) is one of the best-known tumor-associated antigens for CRC [12, 13, 14]. It is expressed in normal mucosal cells of the colon, but overexpressed in adenocarcinomas of the colon. Therefore, its serum-level is elevated in CRC patients [14]. CEACAM5 is an adhesion molecule that was shown to be involved in cell adhesion, migration, anoikis, tumor invasion and metastasis [15, 16]. It furthermore activates inhibitory CEACAM1 signaling in natural killer cells (NK cells) and thereby circumvents cytotoxicity of NK cells [15, 16]. CEACAM6, another family member of the carcinoembryonic antigen family, is already upregulated in benign precursor lesions like hyperplastic colorectal polyps and early adenomas [17]. Moreover,

CEACAM6 is an independent predictor of poor survival [18] and is involved in tissue architecture and colonocyte differentiation [19].

IL-6 was previously shown to increase the expression of CEACAM5 on some CRC cells [20, 21]. However, this relationship was not observed for all CRC cell lines and other studies only found a very small and not significant stimulatory effect of IL-6 on the CEACAM5 expression [22]. To the best of our knowledge, no study has yet examined the relationship between IL-6 trans-signaling and CEACAM5 and CEACAM6.

Thus, the aim of this study was to analyze the impact of IL-6 trans-signaling on the expression of CEACAM5 and CEACAM6.

4.2 Material and Methods

4.2.1 Cell culture and proteins

The human colorectal adenocarcinoma cell lines HT29 (called HT29p for 'parental' cells to distinguish it from other HT29 derivatives in our laboratory) and SW480 were obtained from the American Type Culture Collection (ATCC). HT29c cells had been generated in our laboratory by repeated injection of HT29 cells into the portal venous system of nude rats, subsequent isolation from liver metastases and reculturing *in vitro* [23, 24]. Colo357 cells, derived from a metastasis of a pancreatic adenocarcinoma, were a kind gift of Dr. R. Morgan (Denver, CO) [25]. These cells were routinely cultured in Roswell Park Memorial Institute medium (RPMI)-1640 medium (Gibco/Life Technologies, Darmstadt, Germany) supplemented with 10 % fetal bovine serum (FBS, PAN-Biotech, Aidenbach, Germany), 1 mM sodium pyruvate (Gibco) and 2 mM glutaMAX (Gibco). The human colorectal adenocarcinoma cell line Caco-2 was obtained from ATCC. It was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10 % FBS, 1 mM sodium pyruvate and 2 mM glutaMAX. The normal mucosa-derived colon cell lines CSC1 and NCM460 were a kind gift of Dr. Mary Pat Moyer (San Antonio, TX, USA). These cell lines were maintained in M3 Base cell culture medium complete (M300A-500, Incell, San Antonio, TX, USA) with 10 % FBS. Ba/F3-gp130-mIL-6R are Ba/F3 cells that had been stably transfected with the human IL-6R and gp130. They were a kind gift of Prof. Stefan Rose-John (Kiel, Germany), and were cultured in DMEM high glucose medium (Gibco) supplemented with 10 % FBS, 1 mM sodium pyruvate, 2 mM glutaMAX and 1 ng/ml IL-6 (from Prof. Stefan Rose-John). All cells were maintained at 37 °C in a humid atmosphere with 5 % CO₂ and routinely checked for mycoplasma contamination with the MycoTrace kit (PAA/GE Healthcare, Cölbe, Germany).

IL-6 and Hyper-IL-6 were produced by the group of Stefan Rose-John as previously described [26, 27].

RNA isolation and cDNA synthesis

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured in a Nanodrop spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany) and quality-checked on a 1 % agarose gel. 2 µg of RNA were reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

4.2.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

PCR was performed using the Dream Taq Green Polymerase (Thermo Fisher Scientific). The primer sequences are depicted in table 1, and the following conditions were used: initial denaturation: 95 °C, 2 min; denaturation: 95 °C, 30 sec; annealing: 60 °C, 30 sec; extension: 72 °C, 1 min (40 cycles); final extension: 72 °C, 10 min. The PCR product was analyzed by agarose gel electrophoresis on a 2 % agarose gel.

name	sequence 5'-3'	amplicon [bp] (mRNA/genomic)	reference
hu_mIL6R_For	CATTGCCATTGTTCTGAGGTTC	280/not amplified	[28]
hu_IL6R_Rev	GTGCCACCCAGCCAGCTATC	278(mIL6R),280(sIL6R)/not amplified	[28]
hu_gp130C	GGTACGAATGGCAGCATAACA	713/10170	[29]
hu_gp130D	CTGGACTGGATTTCATGCTGA	713/10170	[29]
hu_IL6_For1	TCCACAAGCGCCTTCGGTCC	621/not amplified	primerblast
hu_IL6_Rev1	TTGCCGAAGAGCCCTCAGGCT	621/not amplified	primerblast
hu_RPL22_For	TCGCTCACCTCCCTTTCTAA	250/6652	[30]
hu_RPL22_Rev	TCACGGTGATCTGCTCTTG	250/6652	[30]

Table 4.1: Primer sequences used for RT-PCR

Quantitative real-time polymerase chain reaction (qPCR)

cDNA was diluted 100-fold in nuclease-free water. 2 µl of diluted cDNA were used in a 20 µl reaction with FastSybr Green mastermix (Applied Biosystems/Life Technologies). The primer sequences are depicted in table 4.2, and the following conditions were used: initial denaturation: 95 °C, 20 sec; denaturation: 95 °C, 3 sec; annealing/extension: 60 °C, 30 sec (usually 40 cycles). Specificity of the product was verified by melt curve analysis and agarose gel electrophoresis.

4.2.3 Phorbol-12-myristate-13-acetate (PMA) stimulation and enzyme-linked immunosorbent assays (ELISAs)

For PMA stimulation, HT29p cells were seeded in a 96-well plate. On the next day, the medium was changed to remove non-adherent or dead cells. After 72 h, the supernatants were collected to measure the baseline (unstimulated) sIL-6R production of the cells (data not shown). Subsequently, the medium was changed, and the cells were stimulated for 2 h at room temperature (RT) with medium containing either 100 nM PMA (Calbiochem/Merck,

name	sequence 5'-3'	amplicon [bp] (mRNA/genomic)	reference
hu_CEA_For	CTTTATCGCCAAAATCACGC	138/6195	http://primerdepot.nci.nih.gov
hu_CEA_Rev	CCAGCTGAGAGACCAGGAGA	138/6195	http://primerdepot.nci.nih.gov
hu_CEACAM6_For1	GCATGTCCCCTGGAAGGA	179/1076	[31]
hu_CEACAM6_Rev1	CGCCTTTGTACCAGCTGTAA	179/1076	[31]
hu_RPL22_For	TCGCTCACCTCCCTTCTAA	250/6652	[30]
hu_RPL22_Rev	TCACGGTGATCTTGCTCTTG	250/6652	[30]
hu_PPIC_For	GGAAAAGTCATTGATGGGATG	127/1907	sequence from Eva Simon, Kiel
hu_PPIC_Rev	CAAAAAGGCGTTTTCACGTCTA	127/1907	sequence from Eva Simon, Kiel
hu_SDHA_For	ATTTGGTGGACAGAGCCTCA	126/not amplified	sequence from Eva Simon, Kiel
hu_SDHA_Rev	CTGGTATCATATCGCAGAGACCT	126/not amplified	sequence from Eva Simon, Kiel

Table 4.2: Primer sequences used for qPCR

Darmstadt, Germany) dissolved in dimethyl sulfoxide (DMSO) or 0.5 % DMSO as solvent control in triplicate wells. Supernatants from the triplicate wells were harvested and centrifuged for 15 min at 16,000 x g and 4 °C to remove cells and cellular debris. The purified supernatants were stored at -80 °C until ELISA analysis. sIL-6R concentrations were measured using ELISA kits (R&D Duoset, R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

4.2.4 Analysis of STAT3 phosphorylation and CEACAM expression in western blots

To analyze the phosphorylation of STAT3 and CEACAM5/6, HT29p cells were seeded in 6-well plates. After 48 h, the medium was replaced by serum-free medium. The next morning, cells were stimulated with different concentrations of IL-6 or Hyper-IL-6. After 15 min (STAT3) or 48 h (CEACAM5/6), the cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and stored at -20 °C until analysis for STAT3 phosphorylation in western blots.

For western blots, the lysates were thawed on ice, sonicated and centrifuged (13,000 rpm, 15 min, 4 °C) to remove cellular debris. Protein concentration was determined with the DC assay (Bio-Rad Laboratories, Munich, Germany). Equal amounts of protein were loaded onto a 4-20 % tris-glycine gel (Life Technologies) and separated by SDS-PAGE. Proteins were blotted on a PVDF membrane (Immobilon-FL; Millipore/Merck, Darmstadt, Germany), blocked with 5 % milk or bovine serum albumin for 1 h at RT and incubated with the primary antibody overnight at 4 °C. Secondary antibody incubation was 1 h at RT. All washes were performed with TBS supplemented with 0.01 % Tween-20. Blots were dried with methanol and scanned in an Odyssey imager (LI-COR, Bad Homburg, Germany). Alternatively, horseradish peroxidase (HRP)-coupled secondary antibodies were used. After incubation, these membranes were incubated with a substrate for electrochemiluminescence (ECL), and readout was performed using films (Amersham Hyperfilm ECL, both GE Healthcare, Munich, Germany) and an Agfa Curix60 developing machine (Agfa, Mortsel, Belgium). The following antibod-

ies were used: P-STAT3 (#9131, Cell Signaling Technology/New England Biolabs, Schwalbach, Germany), STAT3 (#9139, Cell Signaling Technology), β -actin (ab6276, Abcam, Cambridge, UK), goat-anti-mouse-IRDye680 (LI-COR), goat-anti-rabbit-IRDye800CW (LI-COR), CEACAM5 (T84.66, kindly provided by Prof. Neumaier, Mannheim, Germany), CEACAM6 (AM02001PU-N, Acris, Herford, Germany), goat-anti-rabbit IgG-HRP (#7074, Cell Signaling), horse-anti-mouse IgG-HRP (#7076, Cell Signaling).

4.2.5 CEACAM analysis by flow cytometry

For the analysis of CEACAM molecules by flow cytometry, cells were harvested after Accutase treatment (GE Healthcare, Munich, Germany). Subsequently, all steps were performed on ice. Cells were washed with FACS buffer (PBS with 2 % human serum, 2 mM EDTA, 0.02 % sodium azide), blocked with FACS buffer for 15 min and stained with primary antibodies diluted in FACS buffer (30 min on ice). Subsequently, they were washed three times with FACS buffer and incubated with the fluorochrome-coupled secondary antibody. After washing, the cells were incubated with FACS buffer containing 7AAD (BD Bioscience, Franklin Lakes, NJ, USA). Afterwards, the cells were measured in a FACS Calibur (BD). Weasel software v3.0 (chromocyte, Sheffield, UK) was used for data analysis. Dead cells were excluded by gating for the 7AAD-negative cells, as dead cells were previously shown to be false positive for CEACAM5/6. The following antibodies were used: CEACAM5 (C1P83, generated in our laboratory), CEACAM6 (AM02001PU-N, Acris, Herford, Germany), mouse IgG1-isotype control (X0931, Dako, Glostrup, Denmark), anti-mouse-Alexa488 (Invitrogen/Life Technologies, Darmstadt, Germany).

4.2.6 Immunofluorescence staining

Cells were seeded in chamberslides (Life Technologies). When they reached 70-80 % confluency, they were put on ice and rinsed 3x with cold PBS. Cells were fixed for 5 min with methanol at -20 °C. Subsequently, everything was performed at RT. Washing was repeated 3x. Unspecific binding was blocked by incubation with staining buffer (PBS containing 1 % BSA and 1 % horse serum). After washing with PBS, cells were incubated for 1 h with the primary antibody diluted in staining buffer. Washing was repeated three times. Cells were incubated with secondary antibody diluted in staining buffer for 1 h at RT. Washing was repeated three times, and slides were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Pictures were taken with an Axioskop 40 fluorescence microscope (Carl Zeiss, Jena, Germany).

4.3 Results

4.3.1 Colon cell lines express molecules of the IL-6 signaling pathway

To understand the impact of IL-6 on colon cells, at first we analyzed by reverse transcription polymerase chain reaction (RT-PCR), whether normal mucosa-derived colon cell lines and colorectal cancer cell lines express key components of the IL-6 signaling pathway. Most cell lines expressed IL-6. However, the level among the cell lines varied, and the parental HT29 cell line (HT29p) did not show any IL-6 expression (Fig. 4.1 A). Moreover, all cell lines showed expression of the IL-6R and of the co-receptor gp130.

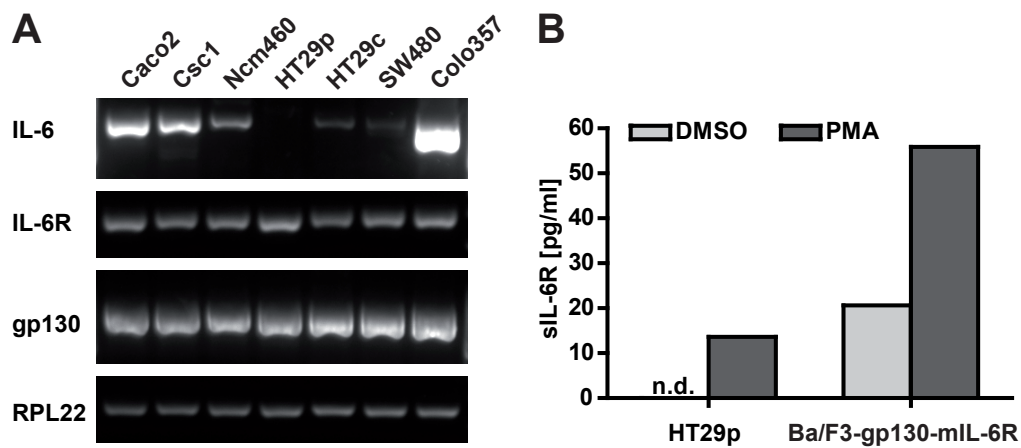


Figure 4.1: Colon cells express IL-6 and the IL-6R. **A**, RNA from different colorectal cancer cells (Caco-2, HT29p, HT29c, SW480) and normal mucosa-derived colon cells (Csc-1, Ncm460) was extracted, reverse-transcribed into cDNA and analyzed for the expression of IL-6, mIL-6R and gp130. The pancreatic cell line Colo357 served as positive control. RPL22 was used to ensure equal transcription of cDNA. **B**, HT29p cells were seeded in 6-well plates. After 72 h, the medium was replaced by medium containing either dimethyl sulfoxide (DMSO) or phorbol-12-myristate-13-acetate (PMA). The supernatants were collected after 2 h and the sIL-6R concentration determined by an ELISA assay. Ba/F3-gp130-mIL-6R cells were used as a positive control. **n.d.** not detected

Yet, we did not detect the IL-6R on the surface of colon cells by flow cytometry (data not shown). Therefore, we assumed that either the expression level was quite low or that the receptor was shed. IL-6R is mainly shed by ADAM17, which is strongly activated by phorbol-12-myristate-13-acetate (PMA) [32]. Stimulation with PMA led to an increase of the sIL-6R concentration in the supernatant (Fig. 4.1 B), demonstrating that colorectal cancer cells express the IL-6R protein on their membrane and that it can be shed from the surface.

4.3.2 CEACAM5 and CEACAM6 are expressed in most colon cell lines

Before we analyzed the relationship between IL-6 and CEACAMs, we examined the expression of CEACAM5 and CEACAM6 in different colorectal cell lines. Most of these cell

lines expressed CEACAM5 as well as CEACAM6. However, the level varied between different cell lines. On mRNA level, HT29p and Ncm460 cells showed the highest expression of CEACAM5 (Fig. 4.2). HT29p also had the highest mRNA level of CEACAM6. Interestingly, the cell line HT29c, which was derived from HT29p cells using a successive intrahepatic selection procedure in rats [23, 24], showed a much lower expression of CEACAM5 and CEACAM6 than HT29p. The cell line Caco-2 clearly increased its expression of CEACAM5 and CEACAM6 with higher confluency. SW480 cells did not express any CEACAM5/6.

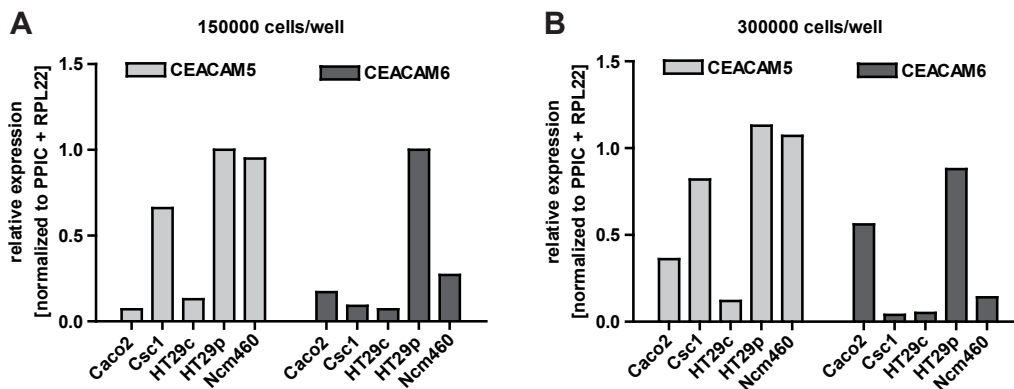


Figure 4.2: Most colon cell lines express CEACAM5 and CEACAM6. A, 150000 or 300000 (B) colorectal cancer cells were seeded in 6-well plates. After 48 h hours the RNA was extracted and analyzed by qPCR for the expression of CEACAM5 and CEACAM6. PPIC and RPL22 were used as reference genes.

On protein level, we analyzed the CEACAM5/6 surface expression by FACS (Fig. 4.3). In a given cell population, only a fraction of cells were positive for CEACAM5 and CEACAM6. As on the mRNA level, HT29p cells had the highest expression level (represented by the difference in the mean fluorescence intensity, Fig. 4.3) as well as the highest percentage of positive cells. Ncm460 cells showed a similar amount of positive cells, but the expression level was lower than in HT29p cells. Again, HT29c cells exhibited a lower expression of CEACAM5/6 than HT29p, and SW480 did not show any surface expression.

Furthermore, we confirmed by immunofluorescence, that only some cells in the population expressed CEACAM5 or CEACAM6 (Fig. 4.4). These positive cells were often found in clusters. But sometimes, single cells were positive and surrounded by negative cells. The CEACAM proteins were not just expressed on the cell membrane but also found in the cytoplasm (Fig. 4.4). These findings are similar to pictures depicted in the *human protein atlas* (<http://www.proteinatlas.org>) for the Caco-2 cell line.

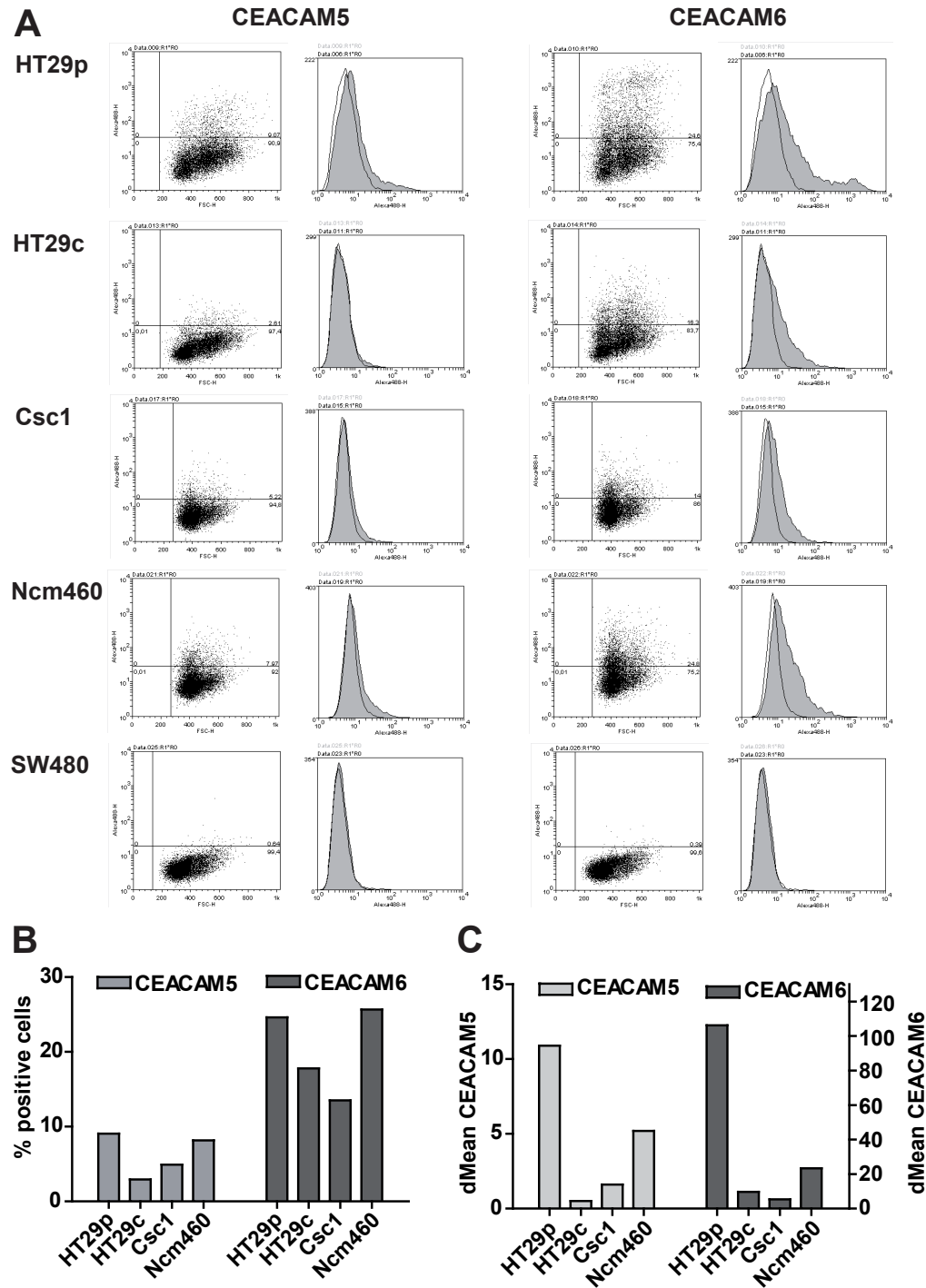


Figure 4.3: Most colon cell lines express CEACAM5/6 on their surface. A, The CEACAM5/6 expression of different colorectal cancer cell lines (HT29p, HT29c, SW480) and normal mucosa-derived colon cell lines (CSC1, NSM460) was analyzed by FACS using specific antibodies (grey) or an unspecific isotype control (white). The amount of positive cells (B) as well as the change in the mean fluorescence intensity (C) were analyzed.

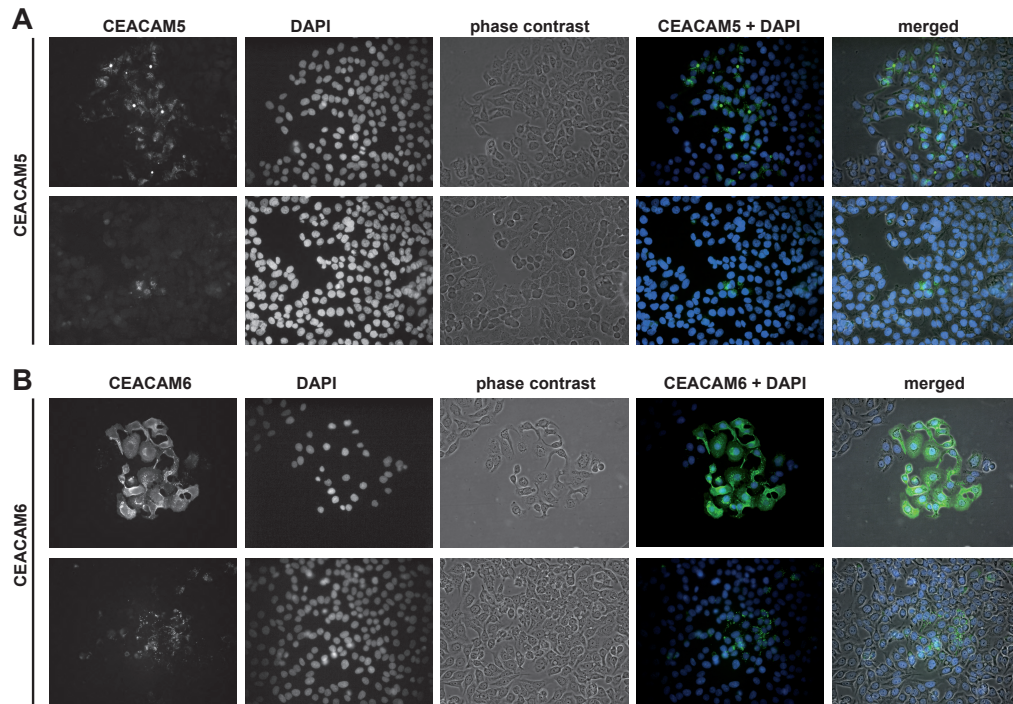


Figure 4.4: CEACAM5 and CEACAM6 are heterogeneously expressed in the culture. The CEACAM5 (A) and CEACAM6 (B) expression of HT29p cells was analyzed by immunofluorescence using CEACAM-specific primary and Alexa488-coupled secondary antibodies (green). DAPI (blue) was used to stain the nuclei and cell morphology was observed by phase contrast microscopy (40x objective). Two representative pictures are shown for each protein.

4.3.3 IL-6 trans-signaling upregulates the expression of CEACAM5 and CEACAM6 in colon cells

To study the effect of IL-6 classic and trans-signaling, HT29p cells were stimulated either with IL-6 or Hyper-IL-6 in different concentrations. We analyzed the phosphorylation of STAT3, as it is one of the earliest steps in the IL-6 signaling cascade. While IL-6 itself only weakly activated STAT3, Hyper-IL-6 led to a strong phosphorylation even at low concentrations (Fig. 4.5). This suggests again that HT29p cells express the IL-6R only in small amounts and are not very responsive to IL-6.

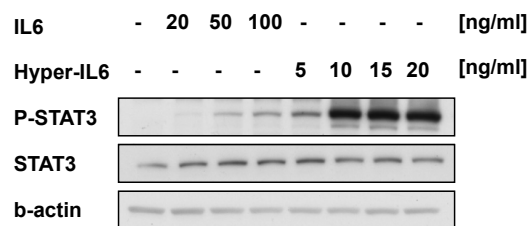


Figure 4.5: Hyper-IL-6 strongly activates STAT3 in HT29p cells. HT29p cells were seeded in 6-well plates, serum-starved overnight and stimulated with different concentrations of IL-6 or Hyper-IL-6. After 15 min, the cells were lysed and the lysates analyzed for STAT3 phosphorylation in western blots.

To answer the question whether IL-6 signaling leads to an upregulation of the CEACAM5/6 expression, we treated HT29p cells with IL-6 [100 ng/ml] or Hyper-IL-6 [15 ng/ml] and analyzed the CEACAM5/6 expression on mRNA level after 6 and 24 h. After 6 h, only slight changes were observed. But after 24 h, the CEACAM5 and CEACAM6 expression were clearly increased by Hyper-IL-6 treatment (Fig. 4.6 A). Yet, IL-6 did not lead to a significant increase in the CEACAM5/6 expression (Fig. 4.6 A).

On protein level, Hyper-IL-6 also strongly increased the CEACAM5 and CEACAM6 expression in different colon cell lines (Fig. 4.6 B). STAT3 was strongly phosphorylated by Hyper-IL-6 in all these cell lines. Treatment with IL-6 led to a much weaker STAT3 phosphorylation although the concentration was higher than of Hyper-IL-6. Consequently, the CEACAM expression was not as strongly increased as with Hyper-IL-6. The cell line SW480 did not express any CEACAM5 and CEACAM6. This did not change after treatment with IL-6 and Hyper-IL-6. This indicates that CEACAM expression is not inducible by IL-6 *de novo*, but typically stimulated by IL-6 trans-signaling (Fig. 4.6 B). While the relationship between IL-6 and the CEACAM5 expression was not clear in the literature, our data suggest that IL-6 leads to a small increase in the expression of CEACAM5 and CEACAM6, but that this increase is much stronger when IL-6 trans-signaling occurs due to the low IL-6R

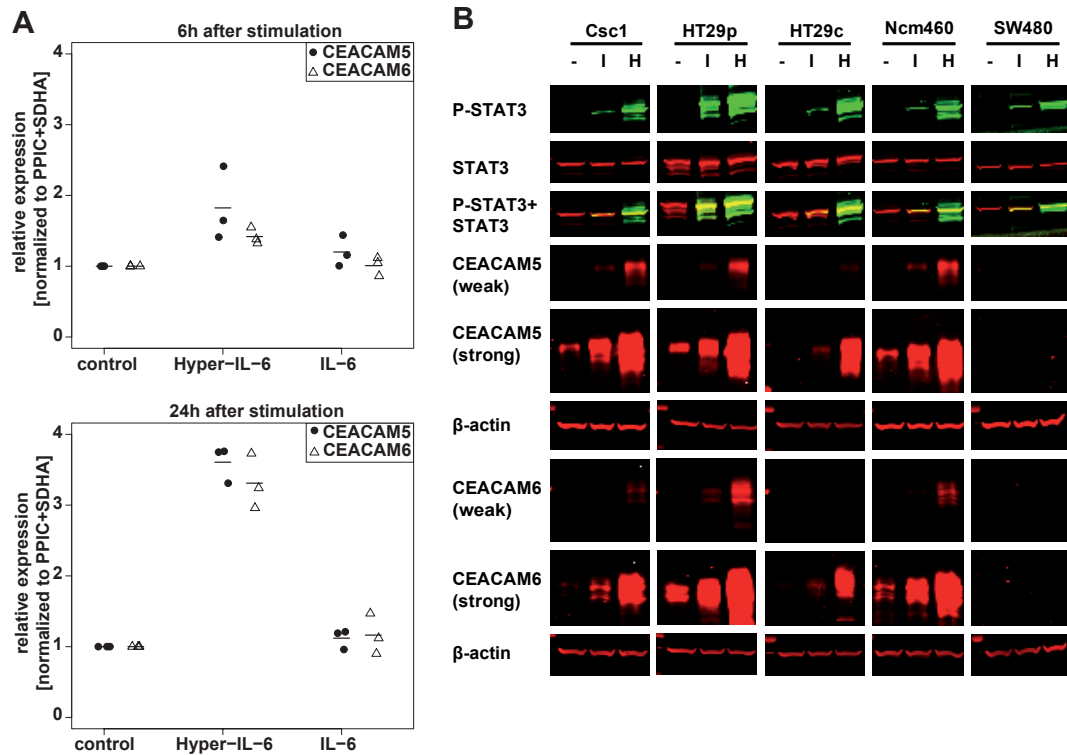


Figure 4.6: Hyper-IL-6 increases the expression of CEACAM5 and CEACAM6. **A**, HT29p cells were seeded in 6-well plates, serum-starved overnight and stimulated with IL-6 [100 ng/ml] or Hyper-IL-6 [15 ng/ml]. After 6 and 24 h, the RNA was isolated and analyzed by qPCR for the expression of CEACAM5 and CEACAM6. PPIC and SDHA were used as reference genes. **B**, Different cell lines were seeded in 6-well plates, serum-starved over night and treated with IL-6 [100 ng/ml] or Hyper-IL-6 [15 ng/ml]. After 15 min (P-STAT3) or 48 h (CEACAMs), the cells were lysed and the lysates analyzed for the phosphorylation of STAT3 and the expression of CEACAM in western blots. β-actin was used to ensure equal protein loading.

expression. Furthermore, we show for the first time that CEACAM6 is upregulated by IL-6 trans-signaling.

4.3.4 The phosphorylation of STAT3 is necessary for the Hyper-IL-6-mediated CEACAM5/6 increase

To analyze, whether the Hyper-IL-6-mediated CEACAM5/6 expression depends on the phosphorylation of STAT3, HT29p cells were pre-treated with FLLL31, a small molecule STAT3 inhibitor derived from curcumin [33]. Subsequently, the cells were stimulated either with normal medium or with Hyper-IL-6. FLLL31 clearly inhibited the Hyper-IL-6-induced phosphorylation of STAT3 (Fig. 4.7 A) and the increase in CEACAM5/6 expression (Fig. 4.7 B).

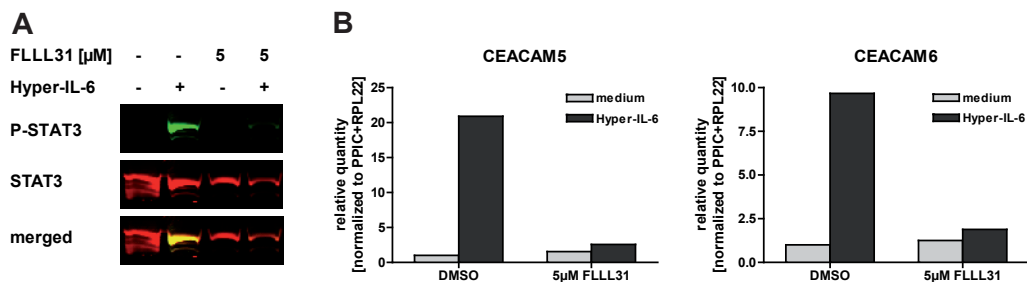


Figure 4.7: Inhibition of STAT3 phosphorylation prevents the Hyper-IL-6 mediated CEACAM increase. **A**, HT29p cells were seeded in 6-well plates, serum-starved overnight and pre-treated with FLLL31 [5 µM] for 2 h. Subsequently, cells were treated with serum-free medium with or without Hyper-IL-6 [15 ng/ml]. After 15 min, the cells were lysed and the lysates analyzed for the phosphorylation of STAT3 in western blots. **B**, After 24 h, the RNA was isolated and analyzed in qPCR for the expression of CEACAM5 and CEACAM6. PPIC and RPL22 were used as reference genes.

4.3.5 IL-6 trans-signaling stabilizes HIF-1 α but does not lead to an upregulation of HIF-1 α target genes

IL-6 was shown to increase the expression of hypoxia-inducible factor 1 α (HIF-1 α) at the protein synthesis level [34]. HIF-1 α , in turn, was described to upregulate CEACAM5 and CEACAM6 [35, 36]. Therefore, we assumed that the (Hyper-)IL-6-mediated CEACAM5/6 increase could be due to an increased HIF-1 α level.

We confirmed that treatment with Hyper-IL-6 increased the HIF-1 α protein level after 6 h of incubation. At later time points this difference decreased (Fig. 4.8 A). On mRNA level, we did not detect a significant upregulation of HIF-1 α mRNA (Fig. 4.8 B). HIF-1 α can be chemically stabilized by the iron chelator deferoxamine mesylate (DFO). DFO inhibits prolyl hydroxylases, which target HIF-1 α through degradation [37]. We used DFO to confirm that HIF-1 α leads to an upregulation of CEACAMs. Treatment of HT29p cells with DFO led to a

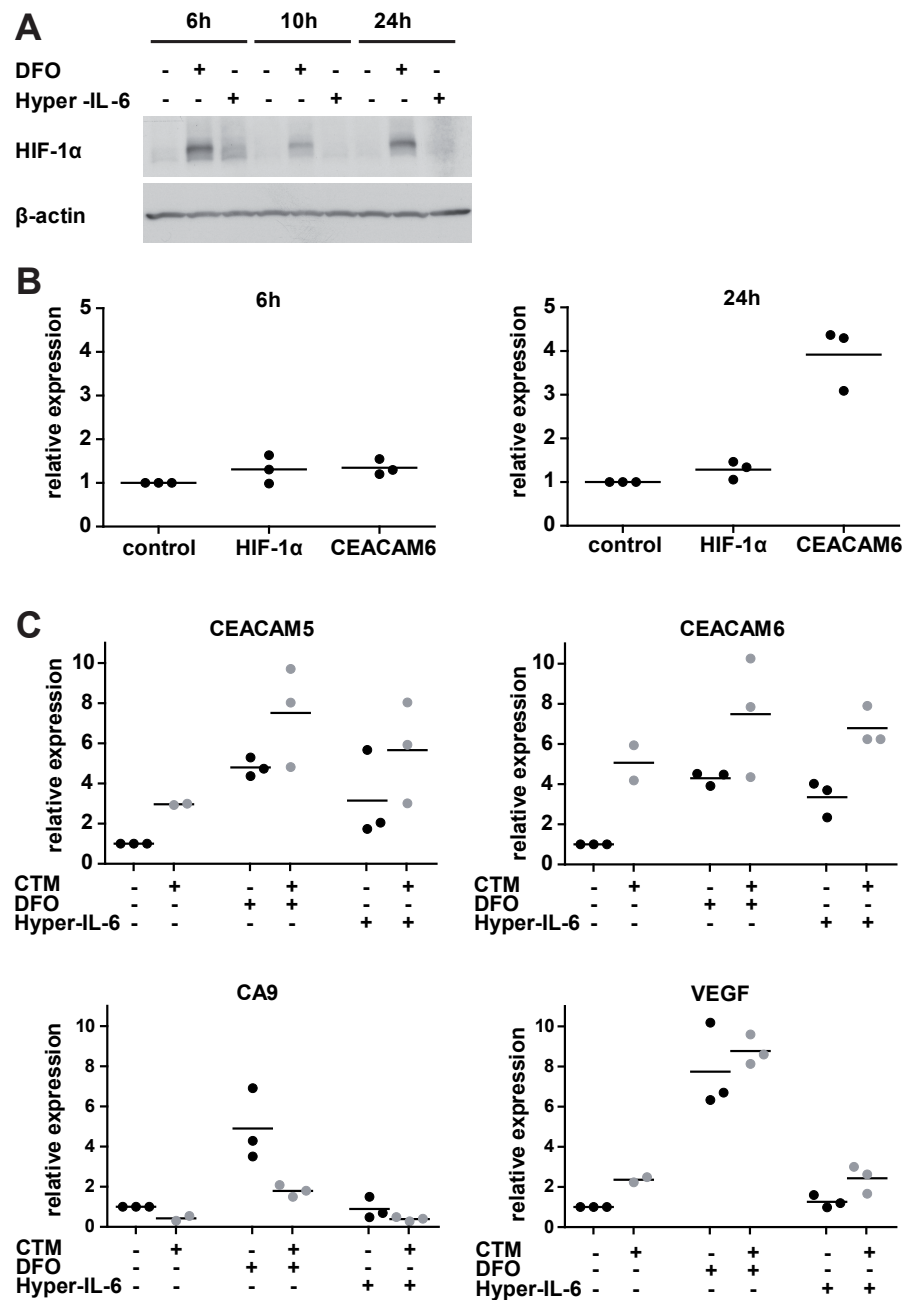


Figure 4.8: Hyper-IL-6 increases the expression of HIF-1 α on protein level. **A**, HT29p cells were treated with Hyper-IL-6 [15 ng/ml] or with 100 μ M of deferoxamine (DFO). Cells were lysed at the indicated time points and analyzed on western blots for the expression of HIF-1 α . DFO served as positive control. **B**, HT29p cells were treated with Hyper-IL-6 [15 ng/ml]. After 6 or 24 h, RNA was extracted and analyzed by qPCR for the expression of HIF-1 α . The expression of CEACAM6 was used as a control. **C**, HT29p cells were seeded in 6-well plates. Cells were serum-starved for 7 h and pre-incubated either with DMSO as solvent control [0.1 %] or with chetomin (CTM, 100 nM). After 4 h, cells were additionally stimulated with DFO [100 μ M] or with Hyper-IL-6 [15 ng/ml]. After 24 h, RNA was extracted and analyzed by qPCR for the expression of CA9, VEGF, CEACAM5 and CEACAM6.

clear upregulation of CEACAM5 as well as of CEACAM6 (Fig. 4.8 C). The classical HIF-1 α target genes CA9 and VEGF were used as positive controls.

To analyze whether the Hyper-IL-6-induced CEACAM5/6-upregulation is mediated via HIF-1 α , we blocked the interaction of HIF-1 α with the transcriptional coactivator p300 by chetomin (CTM). CTM had been identified in a high-throughput screen with >600,000 substances (and secondary analyses) as the only submicromolar inhibitor of HIF-1 α /p300 interaction [38]. This inhibition is mediated by a zinc ejection mechanisms [39]. As a control for effective inhibition, we analyzed the expression of the HIF-1 α target gene CA9 [40]. As expected, CTM decreased the expression of CA9 under control conditions or after DFO exposure. Treatment with Hyper-IL-6 did not lead to an upregulation of CA9 (Fig. 4.8 C).

Interestingly, CTM increased the CEACAM5 and CEACAM6 expression instead of decreasing it. This was the case no matter whether cells were treated with normal medium, DFO or Hyper-IL-6 (Fig. 4.8 C). We found the same pattern for VEGF, which is another well-known HIF-1 α target gene (Fig. 4.8 C). VEGF was also not upregulated by Hyper-IL-6 treatment (Fig. 4.8 C). Further studies are necessary to analyze the role of HIF-1 α in the Hyper-IL-6-mediated CEACAM increase.

4.4 Discussion

In this study, we showed that IL-6 trans-signaling upregulated the expression of CEACAM5 and CEACAM6 in colorectal cancer (CRC) cells. IL-6 trans-signaling is known to be important for the development of CRC [6]. We showed that some CRC cells express IL-6 constitutively, as well as the IL-6R and gp130 on the mRNA level. Yet, cells only weakly responded to stimulation with IL-6 alone. In comparison, IL-6 trans-signaling (induced by Hyper-IL-6) strongly phosphorylated STAT3. Interestingly, also cell lines originally derived from normal mucosa expressed IL-6, IL-6R and gp130. Whether this is also the case with real normal mucosal cells *in vivo* is not clear, as the cell lines have acquired some transformation-associated characteristics over the long time period they have been in culture [41].

IL-6 classic signaling weakly increased the expression of CEACAM5 and CEACAM6 in different colon cell lines. In comparison, IL-6 trans-signaling had a much stronger effect. This is probably due to a low IL-6R expression on the cell surface. However, it could also depend on the cell type, as the pancreatic cancer cell line Colo357 responded much stronger to IL-6 (compare chapter 3.3.1). To better understand and explain the observed effects, the IL-6R expression should be analyzed in a quantitative manner using qPCR or the IL-6 fluorokine kit (R&D systems). The IL-6R expression could also be the explanation why some previous studies described an effect of IL-6 on the CEACAM expression [20, 21] while others did not [22].

IL-6 trans-signaling induced a strong STAT3 phosphorylation and increased the CEACAM5/6 expression. This influence on the CEACAM expression depended on the STAT3 phosphorylation, as an inhibitor of STAT3 phosphorylation blocked the Hyper-IL-6-mediated CEACAM increase. We also observed a Hyper-IL-6-mediated CEACAM5/6 up-regulation in the pancreatic cancer cell line Colo357 (compare chapter 3.5.2).

Moreover, Hyper-IL-6 led to an increase in the level of HIF-1 α . Interestingly, this increase was only observed on the protein level but not on mRNA level, an effect previously described for IL-6. Briggs showed in his dissertation that IL-6 increases the rate of HIF-1 α synthesis (translation) rather than the rate of transcription [42], and STAT3 was shown to inhibit the degradation of HIF-1 α [43]. The increased translation seems to be a common mechanism for the HIF-1 α increase after stimulation with growth/oncogenic stimuli [42, 44, 45].

Stabilization of HIF-1 α by the hypoxia mimetic deferoxamine (DFO) led to an upregulation of CEACAM5 and CEACAM6. This suggested that HIF-1 α might be involved in the Hyper-IL-6-mediated CEACAM5/6 upregulation. Moreover, STAT3 and HIF-1 α were shown to interact in transcriptional complexes to regulate the expression of HIF-1 α target genes [46, 47, 48, 49]. Both transcription factors bind to the transcriptional coactivator p300 [50, 51, 52]. While STAT3 interacts with the bromodomain of p300 [51], HIF-1 α binds to the CH1 domain [52]. This interaction between the CH1 domain and HIF-1 α is inhibited by chetomin. The CH1 domain becomes less structured in the presence of chetomin by a zinc ejection mechanism [39]. Therefore, chetomin probably does not inhibit the interaction between p300 and STAT3. This might explain, why chetomin does not inhibit the upregulation of CEACAM5/6 and VEGF after Hyper-IL-6 stimulation, as activated STAT3 could still be able to recruit p300 to the transcriptional complex. However, it is not clear, why chetomin inhibits the CA9 expression. Transcriptional regulation of HIF-1 α target genes seems to be a complex process, in which all transcription factors are tightly regulated and interact in a complex manner. Therefore, further studies are needed to better understand these interactions.

In summary, we showed in this study that IL-6 trans-signaling increases the expression of CEACAM5 and CEACAM6 in colon cells. This may be important for tumorigenesis, as CEACAM5 and CEACAM6 were shown to be involved in adhesion, migration, invasion and metastasis [16]. Inhibiting IL-6 trans-signaling therefore arises as a promising treatment option for colorectal cancer.

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Chapter 5

Conclusion and Outlook

This work shows that IL-6 inhibition reduces the tumor growth of pancreatic cancer *in vivo* and that IL-6 upregulates cell adhesion molecules of colorectal cancer (CRC) *in vitro*. The results for pancreatic cancer are in line with other studies also suggesting IL-6 as a promising target for the treatment of pancreatic cancer (reviewed in chapter 2). In the present work we specified this approach to IL-6 trans-signaling. Inhibition of IL-6 trans-signaling was sufficient to reduce tumor growth, and additional blockade of classic signaling did not have any advantage. Therefore, blockade of trans-signaling is the preferred target for therapeutic intervention in pancreatic cancer. Further cell biological and *in vivo* approaches are necessary for the elucidation of the mechanism of IL-6 inhibition.

At first, the observed reduction of metastasis by IL-6 treatment *in vivo* could further be elucidated by *in vitro* experiments. Analysis of migration and invasion including cell biological experiments and investigation of adhesion molecules might shed some light on the impact of IL-6 on metastasis. This is of importance, as many pancreatic patients present with metastatic disease [1]. Additionally, a possible decrease of apoptosis resistance by IL-6 inhibition should be analyzed in combination experiments with chemotherapeutics. Often, pancreatic tumors develop resistances to apoptosis-inducing drugs [2, 3], and some studies suggest that the modulation of IL-6 signaling could help to overcome these resistances [4, 5]. Especially, a closer look on combination therapies with gemcitabine *in vivo* might be interesting, because gemcitabine is the standard drug in pancreatic cancer therapy. It is used for adjuvant as well as palliative treatment, but unfortunately many PDACs develop resistances [2, 3]. Different concentrations and time points of treatment with sgp130Fc and gemcitabine have to be evaluated to find out whether inhibition of IL-6 trans-signaling can sensitize an IL-6-expressing cell line for gemcitabine treatment. This should be analyzed in palliative as well as in adjuvant settings. Additionally, IL-6 inhibition before surgery in a neoadjuvant treatment scheme should be examined as it could have a beneficial impact on tumor recurrence and metastasis [6, 7].

A dense stroma surrounding the malignant epithelial cells is a hallmark of pancreatic cancer. This microenvironment provides a milieu favoring the proliferation of malignant cells [8]. Its cellular components include fibroblasts, stellate, endothelial, endocrine, nerve and immune cells [8, 9]. Most of them have the ability to release IL-6 [10]. Especially tumor-associated macrophages appear to be a major source of IL-6 in PDAC [11, 12]. Therefore, the role of the tumor microenvironment in cancer progression should be analyzed with IL-6 as target. Co-culture experiments with macrophages, myofibroblasts and endothelial cells would be interesting to get an idea about the possible cross-talk with components of the microenvironment. On the one hand, it should be analyzed whether co-culture induces the expression of IL-6 in any of these cells and whether blockade of IL-6 classic or trans-signaling changes proliferation, survival or epithelial-mesenchymal transition of the tumor cells. On the other hand, the influence of IL-6 on these non-malignant cells should be analyzed, because we observed that pancreatic tumor cells express IL-6.

To better understand the complex network of interactions in the microenvironment *in vivo*, we plan to inoculate IL-6 positive and negative cells in wild type, IL-6 knockout (KO) and sgp130Fc-transgenic mice and to compare their growth. This model first addresses the question whether the tumor growth is decreased when tumor cells themselves do not express any IL-6. The opposite question is what happens if IL-6-positive tumor cells grow in the pancreatic tissue of an IL-6 KO mice. Also, the tumor growth in a system completely lacking IL-6 could be interesting. The difference between IL-6 classic and trans-signaling can be elucidated by comparing IL-6 KO with sgp130Fc-transgenic mice. The currently planned experiment may provide answers to these questions and has the advantage to be a syngeneic model in immunocompetent mice that provides the opportunity to analyze IL-6 in the context of a functional immune system, which is necessary to understand the interactions of an inflammatory cytokine.

Those additional *in vivo* experiments can help to elucidate possible treatment options for clinical studies. The IL-6 inhibitor tocilizumab is already an approved drug used in the clinic for the treatment of rheumatoid arthritis. Therefore, bioavailability and safety are already proven and the effective concentrations can be assumed from existing data. Promising data of a case report exist. In 2013, Mitsunaga *et al* published a patent "Therapeutic agents for pancreatic cancer" [13], in which a case study was described using tocilizumab in combination with gemcitabine. The patient was a 72-year old man with pancreatic cancer of stage IV. He presented with metastatic lesions in the liver, lymph nodes, and peritoneum and had evident ascites. The inflammation marker CRP was elevated in his blood serum. After treatment with a combination of gemcitabine and tocilizumab, his CRP levels decreased within the normal range and ascitis was remarkably reduced. Primary and metastatic tumor lesions

were reduced by 31 % after 12 weeks [13]. In comparison, the average change in tumor size after 12 weeks of gemcitabine monotherapy is an increase of 36.0 % with no elimination of ascitis [13]. This case report is promising as it describes the successful use of tocilizumab in the treatment of pancreatic cancer in humans. Therefore, clinical studies with more patients should follow as soon as possible. As sgp130Fc also recently successfully passed phase I clinical trials for the development of inflammatory bowel disease (personal communication by Georg Wätzig from CONARIS, Kiel), it should also be tested in clinical trials for pancreatic cancer. sgp130Fc probably has less side effects like opportunistic infections than tocilizumab treatment. In summary, inhibition of IL-6 signaling emerges as a promising new therapeutic option for the treatment of pancreatic cancer.

These suggestions might also be important for the other gastrointestinal cancer we investigated. For colorectal cancer cells, we showed *in vitro* that IL-6 trans-signaling increases the expression of CEACAM5 and CEACAM6. We observed that the phosphorylation of STAT3 is important for this upregulation. However, we do not know if CEACAM5/6 are direct target genes of STAT3 or if this regulation is induced due to a more indirect pathway. This needs to be examined in further studies. At first, bioinformatic approaches can be used to analyze the CEACAM promoter sequences for potential STAT3 binding sites. Reporter assays with deletion mutants of the promoter can indicate which regions of the promoter are necessary for the Hyper-IL-6-induced upregulation of STAT3. For the CEACAM5 promoter, promoter constructs were described by Kokkonen *et al.* that could be useful [14]. For CEACAM6, new constructs would have to be designed. Subsequently, electrophoretic mobility shift assay (EMSA) or chromatin immunoprecipitation (ChIP) assays would give insight about binding of STAT3 to the putative binding sites.

We observed increased levels of hypoxia-inducible factor 1 α (HIF-1 α) in HT29p cells after stimulation with Hyper-IL-6. Not only IL-6 trans-signaling, but also stabilization of HIF-1 α by the hypoxia mimetic deferoxamine (DFO) increased the expression of CEACAM5 and CEACAM6. This indicates an involvement of HIF-1 α in the Hyper-IL-6-mediated CEACAM5/6 upregulation. However, its role could not be clearly identified, as classical HIF-1 α target genes were not upregulated by IL-6 trans-signaling in our system. Moreover, inhibition of the interaction between HIF-1 α and the transcriptional coactivator p300 by chetomin did not inhibit the Hyper-IL-6-mediated CEACAM5/6 upregulation. Therefore, further studies are needed to better understand the role of HIF-1 α . Overexpression of HIF-1 α by plasmids or downregulation of HIF-1 α by siRNA and subsequent activation of STAT3 by IL-6 trans-signaling or co-transfection with plasmids encoding constitutively active STAT3 forms could be a possibility for further analysis of the interaction between STAT3 and HIF-

1 α . Reporter assays with promoter constructs, EMSAs and CHIP assays can be used as read-outs.

In addition to these experiments for the elucidation of the transcriptional regulation, it would be interesting to analyze the functional outcome of Hyper-IL-6-mediated CEACAM5/6 expression. As both proteins were shown to be involved in metastasis [15], invasion and migration should be examined in the next steps. This could first occur in *in vitro* approaches and later in animal experiments. Other interesting aspects are anchorage-independent growth, resistance to gemcitabine and cell-cell interactions [15]. Such type of experiments might then be even more specified by using shRNA-mediated knock-down experimental strategies for CEACAM5/6.

Interestingly, not only IL-6 signaling was shown to increase the expression of CEACAM5, but previous studies also described an IL-6 upregulation by CEACAM5, thus suggesting a stimulatory loop. CEACAM5 exposure significantly increased the IL-6 secretion of blood cells *in vitro* [16]. CEACAM5 can also interact with the CEA receptor (CEAR) expressed by Kupffer cells in the liver, which in turn upregulate IL-6 [17, 18]. Moreover, CEACAM5 from colorectal carcinomas, which is differently glycosylated than normal CEACAM5, binds to DC-SIGN on immature dendritic cells. This interaction strongly enhanced LPS-induced secretion of IL-6 [19]. Taken together, IL-6 and CEACAM5/6 could be part of a vicious circle, in which IL-6 upregulates CEACAM5/6 that in turn increase the production of IL-6. It would be interesting to further study this relationship *in vitro* and *in vivo*.

In summary, this work gave insight into the relationship between inflammation and cancer progression for two prominent and aggressive gastrointestinal tumor entities. It emphasizes the crucial role of IL-6 and provides data for the beneficial use of IL-6 inhibitors as therapeutic options. Especially the inhibition of IL-6 trans-signaling evolves as a promising target for further preclinical and clinical studies particularly in PDAC.

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Erklärung über den Eigenanteil

Bis auf die Kapitel zwei und drei wurde diese Arbeit noch an keiner anderen Stelle veröffentlicht. Kapitel vier wurde nicht veröffentlicht und ist lediglich im Manuskript-Stil geschrieben. Alle darin dargestellten Versuche wurden eigenständig von mir geplant, durchgeführt und ausgewertet. Die erwähnten Co-Autoren stellten Labor-Ressourcen zur Verfügung und hatten zudem betreuende, beratende und korrigierende Funktionen. Der Eigenanteil an den bereits publizierten Manuskripten ist im Folgenden dargestellt.

Kapitel 2: Interleukin-6: A villain in the drama of pancreatic cancer development and progression

Diese Arbeit wurde publiziert in *Hepatobiliary & Pancreatic Diseases International*, Vol 13, No 4, August 15, 2014 (pages 371-380). Ich habe dabei die Literaturrecherche durchgeführt, das Review konzipiert und zum größten Teil verfasst. Freya Goumas hat am Abschnitt 2.2.2 mitgeschrieben und die anderen Teile überarbeitet. Der Anteil der anderen Autoren bestand in der Betreuung, Beratung und im Korrekturlesen.

Kapitel 3: Inhibition of IL-6 signaling significantly reduces primary tumor growth and recurrences in orthotopic xenograft models of pancreatic cancer

Dieses Manuskript wurde publiziert in *International Journal of Cancer*, online 21 Jan 2015 [epub ahead of print], doi: 10.1002/ijc.29445. Ich habe alle *in vitro*-Versuche eigenständig geplant, durchgeführt und ausgewertet. Hilfe bekam ich vom Institut für Pathologie (v.a. Sandra Krüger) bei der Anfertigung der Schnitte und immunhistologischen Färbungen und von Artur Gontarewicz bei der Auswertung dieser Färbungen. An den Tierversuchen, die von Freya Goumas beantragt und geleitet wurden, habe ich praktisch mitgewirkt und anschließend die Daten ausgewertet. Die sonografischen Untersuchungen wurden von Freya Goumas, Carola Heneweer und Susan Jünger geleitet, durchgeführt und ausgewertet. Ich habe dabei am Vor- und Nachbereiten der Tiere (Narkose, Haarentfernung, Aufwachen) mitgewirkt. Das Manuskript habe ich größtenteils eigenständig konzipiert und verfasst.

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass die vorgelegte Dissertation, bis auf die Beratung durch meinen Doktorvater Prof. Kalthoff und die gekennzeichneten Teile der Publikationen, nach Inhalt und Form meine eigene Arbeit ist. Ich versichere, dass diese Abhandlung noch keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen hat. Teile daraus wurden bereits in Fachzeitschriften veröffentlicht. Mein Anteil an diesen Publikationen ist im Abschnitt *Erklärung über den Eigenanteil* dargestellt. Ich versichere, dass diese Dissertation unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist.

Kiel, den 17. März 2015

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