Role of Interleukin-6 receptor in a murine dextran sodium sulfate induced colitis model and expression and functional characterization of novel variants of its beta-receptor gp130

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„Zu sagen was ist, bleibt die revolutionärste Tat“

nach Rosa Luxemburg

CONTENTS

ABSTRACT

Interleukin (IL)-6 is a cytokine which is involved in inflammation as well as in regeneration. Signal-transduction of IL-6 is mediated via a homodimer of glycoprotein 130 (gp130) which associates with a complex of IL-6 and its alpha receptor the IL-6 receptor (IL-6R). Depending on membrane bound or soluble IL-6R (sIL-6R) used for creation of the signaling complex, signaling is referred to as classic or trans-signaling, respectively. Upon formation of the IL-6/(s)IL-6R/gp130 complex, Janus kinases (JAKs) associated with gp130, get autophosphorylated and subsequently phosphorylate gp130. Gp130 mediated signaling results in the activation of signal transducer and activator of transcription (STAT) 3, activation of phosphatidyl inositol 3 kinase (PI3K) and a mitogen activated protein kinase (MAPK) cascade. This doctoral thesis is dedicated to all 3 players of IL-6 signaling. It focusses on the role of IL-6 and IL-6R in inflammatory bowel diseases (IBD) and identifies and analyses novel variants of the IL-6 beta receptor gp130.

The first 4 chapters deal with two novel variants of gp130 and the characterization of IL-30, a novel member of the IL-6 family of cytokines, which signal via a gp130 homodimer. They also include analysis of protein kinase II (CK2), which is indispensable for gp130 mediated activation of STAT3. Gp130 is the signal transducing receptor not only for IL-6, but also for all other members of the IL-6 family of cytokines including IL-30 (also termed protein 28 (p28)). P28 was shown to induce in a complex with Epstein-Barr virus induced protein 3 (EBI3) signal transduction via gp130/WSX1. In the first chapter it is shown that p28 without EBI3 can induce signal transduction via the IL-6R and a gp130 homodimer. Chapter 2 elucidates the effect of a frequently occurring somatic gp130 deletion variant in inflammatory hepatocellular tumors. The deletion variant was stably transduced in IL-6/sIL-6R dependent Ba/F3-gp130 cells which started to proliferate and showed an IL-6/sIL-6R independent STAT3 phosphorylation. Both processes were inhibited by the IL-11 signaling neutralizing gp130 antibody B-P4. In the third chapter, a novel variant of gp130 was found to originate from alternative polyadenylation. Alternative polyadenylation is a process which occurs in competition to splicing of the heteronuclear (hn) ribonucleic acid (RNA). In the messenger (m) RNA a polyadenylated intron remains. Many tyrosine kinase receptors have dominant-negative soluble isoforms originating from this process. In this chapter, it is shown that also gp130, which is a receptor with associated kinases, has an antagonistic soluble isoform formed by alternative polyadenylation and that this process is conserved among many

mammals. Chapter 4 focusses on the role of CK2 in gp130 mediated signal transduction. CK2 is indispensable for STAT3 activation in every kind of gp130 mediated signal transduction, such as classic and trans-signaling of IL-6. It was shown that blockage of CK2 also prevents cytokine-independent proliferation of Ba/F3-gp130 cells transduced with the frequently occurring somatic gp130 deletion variant investigated in chapter 2.

The last chapter of this thesis deals with the role of IL-6 and IL-6R in a murine dextran sodium sulphate (DSS)-induced colitis. Previous studies showed that IL-6 plays a role for susceptibility of mice in this model of disease. It remained elusive whether this was a beneficial or detrimental role. Thus far, there was no report on the role of the IL-6R in DSS-induced colitis. Recently, it has been shown that IL-6 is important for wound healing whereas, surprisingly, the IL-6R plays a minor role. This might indicate a role for other IL-6R ligands in this model. To elucidate if IL-6 increases the susceptibility of DSS challenged mice and to investigate whether the IL-6R plays a role in DSS-induced colitis, animal models were performed. First, it was excluded that a knock out (KO) of the IL-6R on every cell has an effect on the susceptibility, than a tissue specific KO of the IL-6R proved this result. In contrast to this, mice lacking IL-6 signaling were less susceptible to DSS-induced colitis than control mice.

In brief, this doctoral thesis highlights differences between IL-6 signaling and the signaling via the IL-6R in inflammatory bowel diseases and focusses on novel variants of the IL-6 beta receptor gp130.

SUMMARY IN GERMAN

Interleukin-6 ist ein Zytokin, welches sowohl an entzündlichen, als auch an Regenerations-Prozessen beteiligt ist. Die Signalvermittlung von IL-6 erfolgt durch ein Homodimer aus signaltranduzierendem gp130, das mit dem zuvor gebildeten Komplex aus IL-6 und seinem alpha Rezeptor, dem IL-6 Rezeptor, interagiert. Wurde dieser Komplex aus IL-6 und dem membranständigen IL-6R gebildet, so spricht man von *classic signaling*, wurde er von IL-6 und dem löslichen IL-6R gebildet, so spricht man von *trans-signaling*. Nachdem sich nun ein Komplex aus IL-6, IL-6R und gp130 gebildet hat, kommt es zur Autophosphorylierung der mit gp130 assoziierten Janus Kinasen, welche daraufhin gp130 selbst phosphorylieren. Am Ende der gp130 vermittelten Signaltransduktion steht die Aktivierung von STAT3, PI3K und einer MAPK Kaskade. Diese Doktorarbeit widmet sich allen 3 Protagonisten, die an der IL-6 Signaltransduktion beteiligt sind. Sie konzentriert sich vor allem auf die Rolle von IL-6 und dem IL-6R in entzündlichen Darmerkrankungen und identifiziert und analysiert neue Varianten des IL-6 beta Rezeptors gp130.

Die ersten 4 Kapitel beschreiben zwei neue gp130 Varianten und die Charakterisierung von IL-30, einem neuen Zytokin der IL-6 Familie, das eine Signaltransduktion über ein gp130 Homodimer bewirken kann. Ebenso wird die Rolle von CK2 untersucht, die unabdingbar für die gp130 vermittelte STAT3 Aktivierung ist. Gp130 ist der signaltransduzierende Rezeptor für IL-6 und andere Mitglieder der IL-6 Zytokinfamilie wie IL-30 (auch als p28 bezeichnet). Dieses kann einen Komplex mit EBI3 bilden, der über ein Dimer aus gp130 und WSX1 Signale an Zellen vermittelt. In Kapitel 1 wird nachgewiesen, dass p28 auch ohne EBI3 Signale vermitteln kann und dies über den IL-6R und ein Homodimer von gp130 geschieht. Kapitel 2 betrachtet die Wirkung einer häufig vorkommenden somatischen gp130 Deletionsvariante in entzündlichen hepatozellulären Tumoren. Die Deletionsvariante wurde stabil in IL-6/sIL-6R abhängige Ba/F3-gp130 Zellen transduziert, die daraufhin anfingen sich zu teilen und eine IL-6/sIL-6R unabhängige STAT3 Phosphorylierung erfuhren. Beides wurde durch den IL-11 Signal-neutralisierenden gp130 Antikörper B-P4 inhibiert. Im dritten Kapitel wird eine neue gp130 Variante beschrieben, die durch alternative Polyadenylierung entsteht. Dies ist ein Prozess, der parallel und in Konkurrenz zum Spleißen der hnRNA abläuft. In der späteren mRNA verbleibt dabei ein Intron. Viele Tyrosinkinaserezeptoren haben dominant-negative, lösliche Isoformen, die durch den oben beschriebenen Prozess gebildet werden. In diesem Kapitel wird gezeigt, dass auch gp130, ein Rezeptor mit

assoziierten Kinasen, eine antagonistische, lösliche Isoform besitzt, die durch alternative Polyadenylierung entsteht und in vielen Säugetieren konserviert ist. Kapitel 4 beschäftigt sich mit der Rolle von CK2 in der gp130 vermittelten Signaltransduktion. CK2 ist unentbehrlich für die STAT3 Aktivierung bei jeder Art von gp130-vermittelter Signaltransduktion, wie IL-6 *classic* und *trans-signaling*. Es wurde gezeigt, dass die Inhibition von CK2 auch die Zytokin-unabhängige Proliferation von Ba/F3-gp130 Zellen, die mit der häufig vorkommenden somatischen gp130 Deletionsvariante, die in Kapitel 2 untersucht wurde, transduziert wurden, verhindert.

Das letzte Kapitel dieser Arbeit beschäftigt sich mit der Rolle von IL-6 und dem IL-6R in einer DSS-induzierten Colitis in Mäusen. Vorausgehende Studien hatten eine wichtige Rolle von IL-6 für die Anfälligkeit von Mäusen in diesem Krankheitsmodell gezeigt. Jedoch wurde in einer Studie beschrieben, dass sich IL-6 positiv, in einer anderen, dass sich IL-6 negativ auf den Krankheitsverlauf auswirkt. Ferner gab es bis heute keine Studien, die eine Rolle für den IL-6R in einer DSS-induzierten Colitis nachgewiesen oder widerlegt haben. Kürzlich wurde gezeigt, dass IL-6 wichtig für Wundheilungsprozesse ist, der IL-6R hierbei unerwarteter Weise jedoch nur eine untergeordnete Rolle spielt. Dies könnte eine Rolle anderer IL-6R Liganden bedeuten. Um herauszufinden, ob IL-6 die Anfälligkeit der Tiere für eine DSS-induzierte Colitis steigert und ob der IL-6R ebenfalls eine Rolle in diesem Krankheitsmodell spielt, wurde eine DSS-induzierte Colitis durchgeführt. Zunächst wurde ausgeschlossen, dass das Fehlen des IL-6R auf allen Zellen einen Effekt hat, anschließend wurde das Ergebnis für gewebespezifisches Fehlen des Rezeptors bestätigt. Im Gegensatz dazu konnte gezeigt werden, dass Mäuse bei denen die IL-6 Signaltransduktion blockiert wurde, weniger anfällig für eine DSS-induzierte Colitis waren, als die Kontrolltiere.

Diese Doktorarbeit zeigt die Unterschiede zwischen IL-6 Signaltransduktion und der Signaltransduktion über den IL-6R in entzündlichen Darmerkrankungen auf und legt einen besonderen Schwerpunkt auf neue Varianten des IL-6 beta Rezeptors gp130.

1 INTRODUCTION

1.1 OVERVIEW OF STRUCTURE-FUNCTION AND FUNCTIONAL ASPECTS IN THE INTERLEUKIN-6 TYPE CYTOKINE FAMILY

Cytokines are small pleiotropic glycoproteins secreted by a variety of cells for communication [1-3]. Based on structural features and common receptor chains for signaling, they are grouped into different families. The Interleukin (IL)-6 family of cytokines shares a four α-helix bundle motif in an up-up-down-down orientation [4] and can transduce its signals via glycoprotein 130 (gp130), leukemia inhibitory factor receptor (LIFR), gp130-like-receptor (GPL), WSX-1 or oncostatin M receptor (OSMR). The family comprises IL-6, IL-11, IL-27, IL-30, IL-31, cardiotrophin-1 (CT-1), cardiotrophin like cytokine (CLC), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), neuropoietin (NPN) and oncostatin M (OSM) [5-8]. IL-6 binds to the soluble or membrane bound, non-signal-transducing IL-6 receptor α (sIL-6R, IL-6R). Subsequently the complex of IL-6/sIL-6R or IL-6/IL-6R binds to the membrane bound signal-transducing β receptor gp130, referred to as trans- or classic signaling, respectively (Figure 1A, B) [9].

The signal-transducing receptor gp130 is ubiquitously expressed [10]. The IL-6R is expressed on leukocytes and hepatocytes, albeit to a lesser extend also in enterocytes for instance [11-14]. It exists as a membrane bound and soluble isoform. Signaling via a gp130 homodimer activated by IL-6/IL-6R is termed classic signaling and is restricted to cells expressing the IL-6R. Signaling activated by IL-6/sIL-6R is termed trans-signaling and renders cells lacking the membrane bound IL-6R responsive for IL-6 signals (Figure 1B). sIL-6R is created by differential splicing [15] and ectodomain shedding by the metalloproteinases A Disintegrin And Metalloproteinase 10 (ADAM10) or 17 (ADAM17) [16-18].

The first identified member of the IL-6 family of cytokines was IL-6. Initially IL-6 was found to induce the final maturation of B cells to antibody producing plasma cells and was therefore named B cell differentiation factor 2 (BSF-2) [19,20]. IL-6 has different effects ranging from development and differentiation of cells as well as expression of various acute phase response genes to stimulation of hepatocytes, osteoclasts and immune cells (Figure 2) [21-25]. It is also secreted by active muscles having a hormone-like function like mobilizing extracellular substrates [3,26].

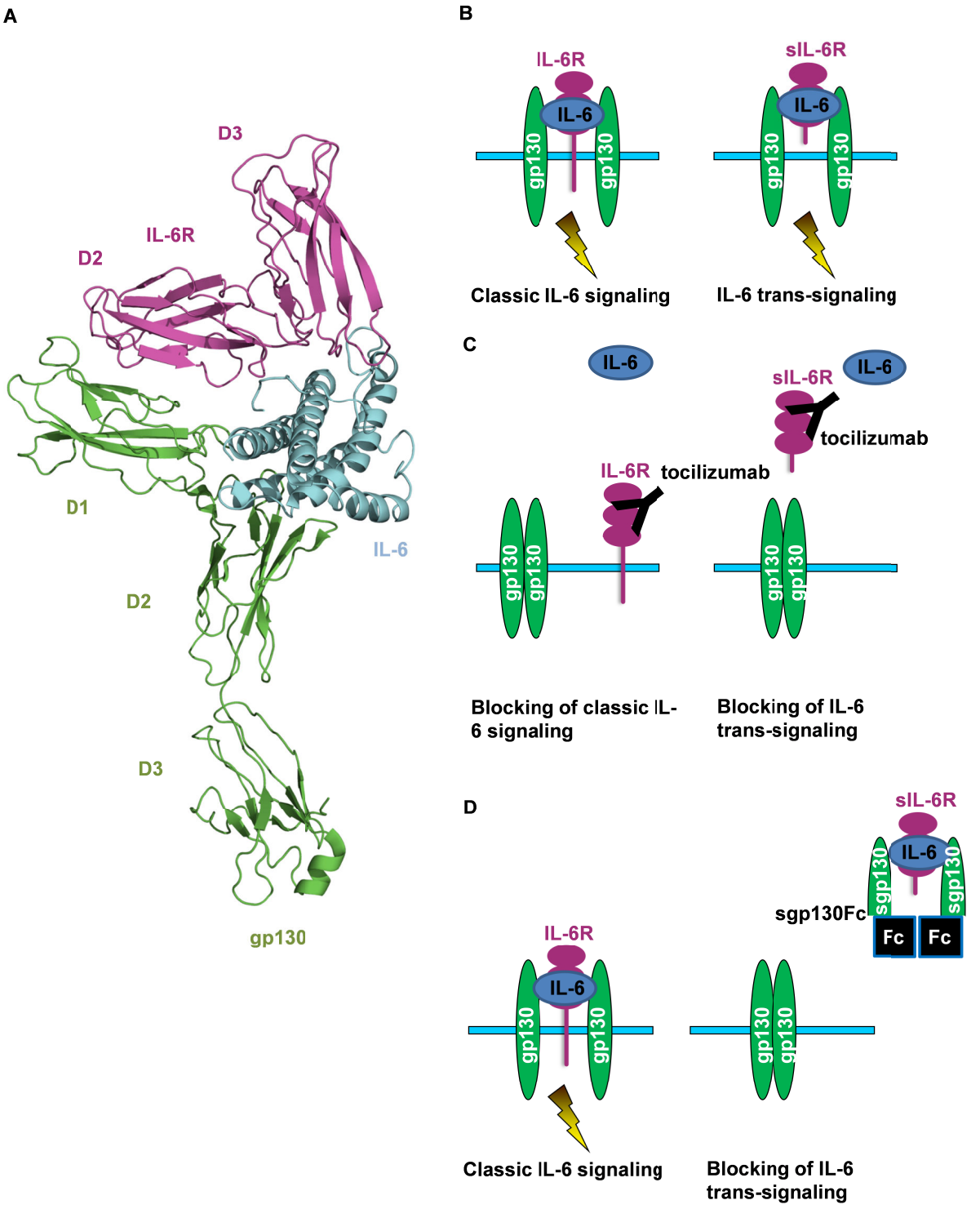

Figure 1: Structure of the IL-6/(s)IL-6R/gp130 complex and possibilities to block IL-6 signaling.
A) IL-6 binds to domain 2 and 3 of the membrane bound or the soluble IL-6R. The IL-6/(s)IL-6R complex subsequently binds to domain 1 and 2/3 of gp130. Domain 4 to 6, the transmembrane region and the intracellular domain are not shown [27]. **B)** The complex of IL-6/IL-6R binds to a homodimer of gp130 leading to signal transduction termed IL-6 classic signaling. Also the complex of IL-6/sIL-6R binds to a homodimer of gp130, termed IL-6 trans-signaling [28]. **C)** The monoclonal antibody tocilizumab is directed against the IL-6R and blocks classic and trans-signaling [29]. **D)** Sgp130Fc is a soluble variant of the membrane bound gp130 fused to a human Fc-tag. The Fc-tag leads to dimerization of two sgp130Fc molecules. The sgp130Fc homodimer competes with the membrane bound gp130 for the binding to the IL-6/sIL-6R complex and thereby blocks IL-6 trans-signaling [29].

IL-6 is known to be involved in several inflammatory disorders, including rheumatoid arthritis (RA), Castleman's disease and inflammatory bowel diseases (IBD) [30,31], thus blockade of IL-6 signals is an approach for treatment of several inflammatory disorders. Global blockade of IL-6 classic and trans-signaling by the IL-6R neutralizing antibody tocilizumab is approved in the US, EU and Japan for the treatment of RA and other diseases (Figure 1C). Blocking IL-6 trans-signaling was shown to ameliorate chronic inflammatory diseases [29]. Classic signaling is important for the induction of the hepatic acute phase response, it inhibits epithelial cell apoptosis and in some studies it was shown to play a role in intestinal epithelial cell proliferation [28]. Trans-signaling is important for recruitment of mononuclear cells, inhibition of T cell apoptosis and inhibition of regulatory T cell (T_{reg}) differentiation [28]. Therefore blockade of IL-6 trans-signaling by soluble variants of gp130 might offer an attractive alternative treatment option by leaving IL-6 classic signaling intact (Figure 1D) [32]. Soluble gp130 (sgp130) variants only bind to IL-6, which is in a complex with sIL-6R. In an excess of IL-6 compared to sIL-6R, not all IL-6 molecules can be neutralized by sgp130 variants. Free IL-6, which is not in a complex with sIL-6R, can still induce IL-6 classic signaling [28,32]. An optimized variant of the trans-signaling inhibitor sgp130Fc is currently in clinical trials.

In the first chapter of this thesis, IL-30 is characterized. We found that IL-30 has agonistic properties and induces signal transduction via a homodimer of gp130. At low concentrations the IL-6R is required for induction of signal transduction, at high concentrations, IL-30 without the IL-6R can recruit and activate a homodimer of gp130 [33].

1.2 GLYCOPROTEIN 130

Gp130, the signal transducing IL-6Rβ, is a type 1 transmembrane protein belonging to the class 1 cytokine receptors with a conserved cytokine binding module (CBM) [34]. The mature human gp130 consists of 896 amino acids (aa) [35] and has a molecular weight of about 130 kDa depending on its glycosylation [36]. Gp130 consists of 6 extracellular domains, a transmembrane and an intracellular domain [34,35]. Domain 1 is an Immunoglobulin (Ig)-like domain, domain 2 to 6 are Fibronectin (FN) III-like domains [35,37].

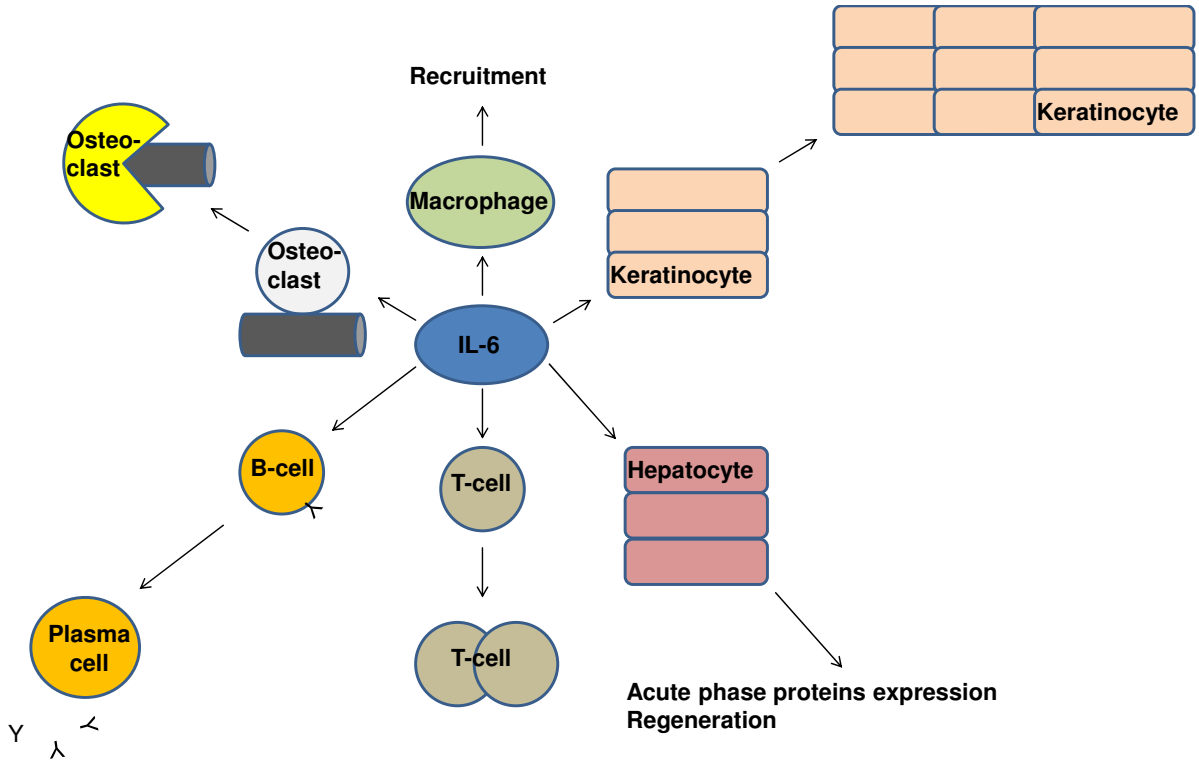

Figure 2: Effects of IL-6 signaling on different cell types.
IL-6 signaling on B-cells leads to differentiation of B-cells to antibody-secreting plasma cells [19,20]. IL-6 signaling on T cells results in T cell differentiation and in the induction of anti-apoptotic genes and thereby cell survival [28]. Hepatocytes produce acute phase proteins upon IL-6 stimulus [23]. IL-6 trans-signaling on hepatocytes causes liver regeneration [25]. On keratinocytes, IL-6 induces proliferation and regeneration [38]. In inflammation, IL-6 trans-signaling via shed neutrophil IL-6R on macrophages, leads to their recruitment and resolution of the inflammatory state [21,39]. Osteoclasts are activated by IL-6 signaling to resorb boney tissue [22].

1.2.1 IL-6 signal transduction via a gp130 homodimer induces STAT3 phosphorylation, a MAPK cascade and PI3K activation

Gp130 mediated IL-6 signal transduction starts with the binding of the complex of IL-6/IL-6R or IL-6/sIL-6R to domain 1 and 2/3 of a gp130 homodimer (Figure 1A) [40]. The intracellular part of gp130 comprises 6 tyrosine residues and conserved box motifs 1 and 2 for recruitment of receptor-associated tyrosine kinases, termed Janus kinases (JAKs). Upon binding of the complex of IL-6/IL-6R or IL-6/sIL-6R to a homodimer of gp130 [40], JAKs get autophosphorylated and subsequently phosphorylate intracellular tyrosine residues of gp130. Some of these residues are part of conserved YXXQ motifs, which serve as binding sites for signal transducer and activator of transcription (STAT) 3 proteins [41]. STAT3 proteins get tyrosine-phosphorylated at Tyr^{705} by the Janus kinases, dimerize and translocate into the nucleus. In the nucleus, the STAT3 homodimer binds to acute phase responsive elements (APREs) and induces transcription of acute phase proteins in the liver and of negative

regulators of the JAK/STAT pathway, including suppressors of cytokine signaling (SOCS) [42,43].

Another pathway which is activated upon gp130 stimulus is the mitogen activated protein kinase (MAPK) pathway. JAK activation leads to phosphorylation of gp130 at Tyr^{759} which becomes a binding site for Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2). This pathway results in the activation of extracellular-signal-regulated kinase (ERK) [44,45].

Gp130 mediated signaling also activates phosphatidyl inositol 3 kinase (PI3K) in a JAK dependent, but tyrosine phosphorylated gp130 independent manner [46].

In chapter 4 of this thesis, protein kinase II (formerly termed casein kinase II (CK2)) is shown to be indispensable for gp130 mediated signal transduction. CK2 is a ubiquitously expressed serine/threonine protein kinase phosphorylating a wide variety of more than 300 substrates [47,48]. Interestingly, genetic knock out (KO) of CK2 in mice is embryonically lethal [49-51].

1.2.2 Pathogenesis of natural occurring and artificial mutations in gp130

Gp130 is the commonly used receptor chain for signal transduction by members of the IL-6 family of cytokines [52]. Therefore one can assume that alterations in expression of gp130 or mutations alter normal cell functions dramatically. Mice deficient for gp130 have been created, but the KO of gp130 was embryonically lethal. Embryos progressively died starting 12.5 days postcoitum, showing defects in heart architecture, reduced numbers of pluripotent and committed hematopoietic progenitors in the liver and differentiated lineages, as well as problems with erythrocyte formation (Table 1) [53]. Conditional deletion of gp130 results in defects similar to the ones of lethal complete KO mice, but also includes neurological defects (Table 1) [54,55].

gp130 variant	Signaling target	Signaling effect	Phenotype
gp130 KO	Complete gp130 signaling	- No signaling via gp130	- Embryonically lethal - Heart architecture defects - Reduced numbers of hematopoietic progenitors in the liver - Problems in erythrocyte formation
Conditional gp130 KO	Complete gp130 signaling	- No signaling via gp130	- Same defects as gp130 KO - Additional neurological defects
gp130ΔSTAT	STAT activation	- No STAT activation - Increased activation of MAPK - Loss of SOCS induction	- Increased numbers of immature colony-forming unit spleen progenitor cells - Elevated numbers of committed myeloid progenitor cells - Leukocytosis - Defects in platelet formation - Degenerative joint disease - Gastrointestinal ulcerations - Failure of uterine implantation - Impaired mucosal and humoral immunity - Diminished acute phase response in the liver
gp130F759	MAPK cascade activation	- Lack of MAPK cascade induction - Loss of a negative regulation of STAT3 signaling (loss of SOCS3 binding site) - STAT3 hyperactivation	- Autoantibody production - Impaired thymic negative selection - Increased memory and activated T cells - Impaired peripheral clonal deletion of T cells - Spontaneous, lymphocyte-dependent development of RA-like autoimmune disease
gp130ΔYY	Ligand independent gp130 activation	- IL-11-like signaling - STAT3 activation devoid of MAPK activation or SOCS3 induction	- Ligand independent cell proliferation - In the liver: Formation of IHCAs

Table 1: Comparison of gp130 variants.
Comparison of gp130 deficient and conditional gp130 deficient mice and gp130ΔSTAT, gp130F759 and gp130ΔYY variants. Signaling targeted by the different variants and the effect on signaling is indicated, as well as the phenotypical outcome in mice or cells [41,43,53-63].

To further differentiate between effects of gp130 KO due to loss of STAT signaling versus MAPK cascade activation, two different KO mice have been generated. The first KO mouse, harboring gp130 truncation leading to loss of all STAT binding sites while leaving the MAPK pathway intact (*gp130ΔSTAT*), shows increased numbers of immature colony-forming unit spleen progenitor cells in the bone marrow and spleen, elevated numbers of committed myeloid progenitor cells in the spleen and peripheral blood, and leukocytosis [56]. Its number of circulating platelets is reduced by 30%. Platelets are also smaller than those of wild type (wt) mice. Administration of either of the gp130 homodimer agonists IL-6 and IL-11 fails to increase platelet numbers, but increases the production of megakaryocytes. This gp130 variant accents the important role of gp130 mediated STAT activation for normal hematopoietic homeostasis and platelet maturation from megakaryocyte progenitors [56]. Beside these effects, the mouse develops degenerative joint disease, gastrointestinal ulcerations and fails uterine implantation. Gastrointestinal ulcerations highlight the importance for STAT activation in intestinal homeostasis. Loss of gp130 dependent STAT activation does not only cause ulcerations, but also impaired mucosal and humoral immunity. Failure of uterine implantation is only dependent on the maternal genotype. In homozygote *gp130ΔSTAT* mice, blastocysts cannot develop. They can be rescued by transferring them to wt or heterozygous pseudo-pregnant mice. Homozygous *gp130ΔSTAT* mice also show a diminished acute phase response in the liver. Interestingly, the mice show an increased activation of the SHP-2 dependent MAPK cascade due to loss of STAT3 dependent induction of SOCS proteins as negative regulators of gp130 signaling (Table 1) [57].

The second KO mouse, harboring a homozygote mutation of gp130 with an exchange of Tyr^{759} to Phe^{759} in gp130 (*gp130*$^{F759/F759}$), shows severe immunological abnormalities, like autoantibody production, impaired thymic negative selection, increased memory and activated T cells and impaired peripheral clonal deletion. These mice also spontaneously develop RA-like autoimmune disease at about 1 year of age. Interestingly, the development of RA-like autoimmune disease is completely dependent on lymphocytes since introduction of the *gp130*$^{F759/F759}$ mutation in recombination-activating gene 2 (RAG-2)-deficient mice, which lack lymphocytes, does not cause disease. These effects are due to loss of a negative regulation of STAT3 signaling, since Tyr^{759} is within the binding site at gp130 for the negative regulator of STAT3 activation SOCS3, resulting in STAT3 hyperactivation. It is also the binding site for SHP-2 activating the MAPK cascade and thereby negatively regulating STAT3 activation (Table 1) [43,58,62,63].

Taken together, gp130ΔSTAT lacks STAT signaling and has an increased MAPK cascade activation, gp130F759 lacks the MAPK cascade activation and has an increased STAT activation. The first highlights the importance for gp130 dependent STAT activation for intestinal and hematopoietic homeostasis, while the second one demonstrates negative effects in T cells of an excessive STAT activation such as loss of self-tolerance and spontaneous development of RA-like autoimmune disease (Table 1) [41,57].

Recently, it has been found that 60% of inflammatory hepatocellular adenomas (IHCAs) exhibit constitutive phosphorylation of STAT3 [59]. IHCA are a subtype of hepatocellular adenoma, a rare benign liver tumor, harboring gp130 gain-of-function somatic mutations leading to receptor activation in the absence of ligands [60]. Nearly all of these mutations are small in-frame deletions within the binding site II of domain 2 (D2) of gp130 [59]. Interestingly, 12% of the remaining 40% of IHCA without mutations in gp130 carried activating STAT3 mutations [60] underlining the importance of STAT3 activation in this type of tumor. Upon IL-6 signaling via gp130, also SOCS proteins which negatively regulate gp130 activation are induced [42,43]. Sustained ligand-independent activation of gp130 is not suppressed by negative feedback loops [64,65]. In Chapter 2 of this thesis, we co-expressed mutant gp130 receptor with a deletion in the domain 2 from Tyr^{186} to Tyr^{190} (ΔYY) which is included in most occurring in-frame deletion variants [59] along with wt gp130. Transduced Ba/F3-gp130-gp130ΔYY cells showed a ligand-independent cell proliferation and STAT3 phosphorylation (Table 1) [61]. This result was consistent with the findings for IHCAs where the mutation of gp130 in the cells is heterozygous as well [59]. Furthermore, we demonstrated, that the neutralizing gp130 antibody B-P4, which specifically inhibits IL-11 mediated signaling via gp130, can inhibit ligand-independent proliferation of Ba/F3-gp130-gp130ΔYY cells.

1.2.3 Soluble gp130 isoforms are generated by differential splicing and alternative polyadenylation

Beside membrane bound gp130, there are soluble isoforms of gp130 which compete with the membrane bound gp130 for the binding to the complex of IL-6/sIL-6R, thereby specifically antagonizing IL-6 trans-signaling [66]. Blocking IL-6 trans-signaling was shown to ameliorate chronic inflammatory diseases [29]. About 400 ng/ml sgp130 is found in human serum [67], but the cellular origin and the mechanism of its generation are poorly understood. So far, three soluble gp130 receptor isoforms of about 110, 90 and 50 kDa have been identified in serum or urine [67-69]. The protein corresponding to the differentially spliced

messenger (m) ribonucleic acid (RNA) of the smallest isoform sgp130-rheumatoid arthritis antigenic peptide-bearing soluble form (RAPS) was verified by Western blotting using specific antibodies directed against the novel C-terminus of sgp130-RAPS [68,70,71]. Sgp130-RAPS is present in blood samples of healthy human donors. Sgp130-RAPS inhibits IL-6 activity. This effect is neutralized by antibodies against sgp130-RAPS [68,72]. In RA, antibodies against this soluble gp130 isoform are detected, but not in patients with other autoimmune diseases or healthy people. Furthermore, the amount of antibodies against sgp130-RAPS is positively correlating with diseases severity. Serum IL-6 concentrations are also positively correlating with disease severity of RA. RA is characterized by synovial inflammation and hyperplasia ("swelling"), autoantibody production (rheumatoid factor and anti–citrullinated protein antibody), cartilage and bone destruction ("deformity"), and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders [73]. It is well accepted that RA involves a complex interplay among genotype and environmental triggers [73].

Two additional differentially spliced gp130 cDNAs were described, but have not been shown to be endogenously translated into soluble gp130 proteins [70,71]. One of these variants (sgp130-Sharkey) was found to be expressed on mRNA level in embryonic tissue. In sgp130-Sharkey, the sequence encoding the transmembrane domain is spliced [70]. The other variant (sgp130-Diamant) was found in blood mononuclear cells by reverse transcription polymerase chain reaction (RT-PCR) on mRNA level. The mRNA had an 85-bp exon inserted in the sequence encoding the extracellular part of the receptor. This results in a frame-shift leading to a stop codon, 1 bp before the beginning of the transmembrane coding part of the mRNA. On genomic level, the novel exon is flanked by introns [71]. The expected size of these variants would, if at all, correspond to the largest endogenous sgp130 variant of about 110 kDa [67,69].

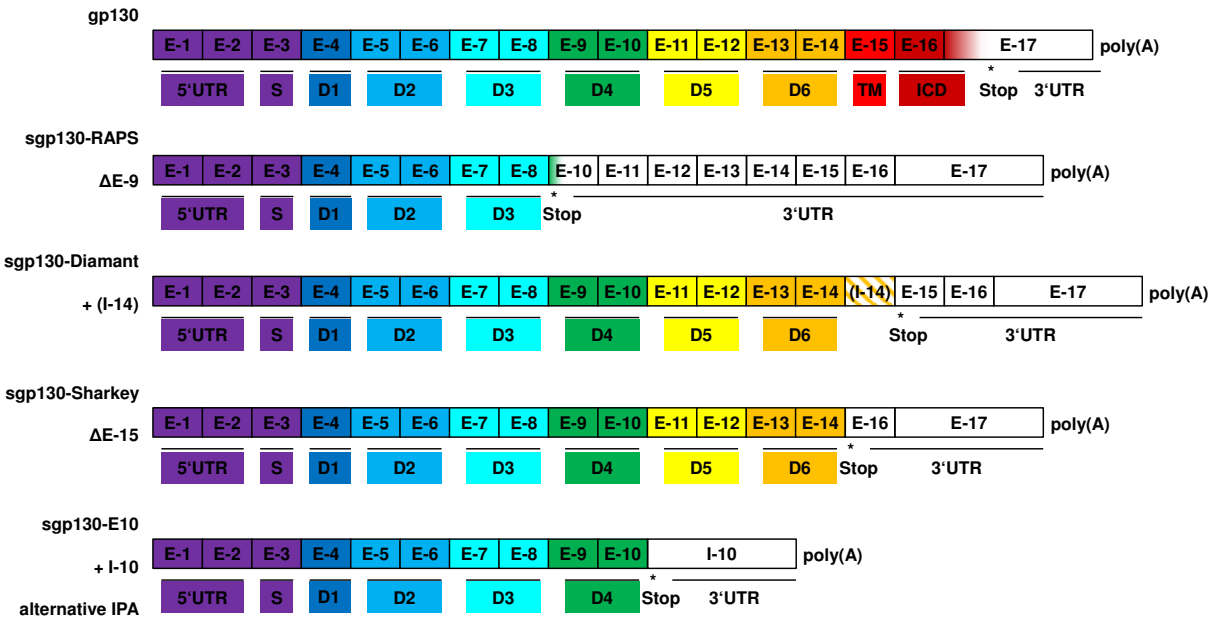

Figure 3: Different mRNA variants for gp130.
For gp130 several mRNA variants are known. The full-length transcript consists of 17 exons (E) which are indicated in boxes. The resulting part of the protein is marked under lines. Exon 1 and 2 are the 5' untranslated region (5'UTR), exon 3 is the signal peptide (S) of gp130. Domain (D) 1 is encoded by exon 4, domain 2 by exon 5 and 6, domain 3 by exon 7 and 8, domain 4 by exon 9 and 10, domain 5 by exon 11 and 12, domain 6 by exon 13 and 14. Exon 15 encodes the transmembrane domain (TM) and exon 16 and 17 the intracellular domain (ICD). In exon 17 there is the stop codon, followed by the 3'UTR. The polyadenylation after exon 17 ends the mRNA [74]. Sgp130-RAPS is the only soluble variant of gp130 which has not only been described on mRNA level, but has also been shown to exist on protein level in the serum. It is formed by alternative splicing of exon 9, leading to a frame-shift and an alternative stop codon in exon 10. The following sequence turns to 3'UTR. The resulting protein lacks domain 4, 5 and 6, as well as the TM and ICD, resulting in a soluble isoform [68]. Sgp130-Diamant has been found on mRNA level. Here, an alternative exon consisting of a part of intron 14 ((I-14)) remains in the mRNA after splicing. This causes a frame-shift and an alternative stop codon in exon 15 which terminates translation just before the sequence encoding for the TM starts. This leads to a soluble isoform comprising the complete extracellular part of gp130 [71]. Sgp130-Sharkey is formed by splicing exon 15 and thereby the TM. Due to a frame-shift, an alternative stop codon in exon 16 is created leading to a soluble isoform with an expected size next to sgp130-Diamant. Sgp130-Sharkey has also been found only on mRNA level [70]. Sgp130-E10 is a novel soluble dominant-negative isoform of gp130 created by alternative polyadenylation. Intron 10 harbors an alternative stop codon and an alternative polyadenylation site which is used for termination of transcription. This process leads to a soluble isoform of gp130 lacking D5, D6, TM and ICD.

It is unknown whether sgp130 variants are also generated by ectodomain shedding of the transmembrane gp130 receptor. Besides differential splicing, alternative intronic polyadenylation (IPA) sites have been identified in many genes [75]. Usage of an intronic polyadenylation site (PAS) leads to shortened mature mRNAs and thereby to truncated protein isoforms. If the C-terminal part of a full-length protein is important for its function, intronic polyadenylation might abolish or alter the function of the shortened protein isoform. Recently, soluble dominant-negative receptor-tyrosine kinase isoforms have been identified which originated from alternative IPA [76]. In chapter 3 of this thesis, a novel sgp130 mRNA which results from alternative IPA in intron 10 is described. The resulting isoform (sgp130-E10) consists of the four N-terminal domains of gp130 and ends in the loop region between

domain 4 and 5 (Figure 3). The sgp130-E10 protein is expressed in peripheral blood mononuclear cells (PBMCs). Furthermore, we expressed sgp130-E10 as myc-His- or Fc-tagged variants and showed that sgp130-E10 specifically inhibits IL-6 trans-signaling.

1.3 ETIOLOGY OF INFLAMMATORY BOWEL DISEASES

Autoimmune diseases comprise a variety of different malignancies which share as a common feature an immune system reacting against its own body. There are two distinct forms of IBD: Crohn's disease (CD) and ulcerative colitis (UC). They are characterized by chronic inflammation of the intestinal tissue and progressive destruction of mucosal integrity [77]. In North America UC has a prevalence of 150 to 300 cases per 100,000 inhabitants while the incidence is between 2.5 to 20 cases per 100,000 inhabitants per year [78]. Most of the patients suffering from IBD develop the disease between the age of 25 and 35 years, but about 20-25% of patients are diagnosed to have IBD during childhood [79]. The predominant symptoms of patients suffering from CD are abdominal pain, diarrhea and weight loss [80] whereas those suffering from UC always have bloody diarrhea and, depending on the extent and severity of disease, abdominal pain, fever, malaise and weight loss [81]. CD normally occurs in the ileum, but can also affect the entire digestive tract from the mouth to the anus, whereas UC always involves the rectum and sometimes the entire colon [82].

A long-term consequence of IBD is tumor formation. A long duration of disease, regardless of its clinical activity, a young age of onset, severe inflammation and a family history of colorectal cancer (CRC) are all risk factors for the development of colon cancer [81]. The 25-year cumulative risk for CRC formation among patients with UC is about 30%, whereas the 25-year cumulative risk for CRC formation among patients with CD is only about 5% [83]. Besides these events in the digestive tract, there are also extra-intestinal manifestations in IBD [81]. One of the most common extraintestinal manifestations is arthritis, including peripheral arthritis and ankylosing spondylitis. Arthritis affects the large joints such as the knees, hips and the spine [80]. Another complication affects the eyes. It derives from the inflammation itself or from the treatment of IBD with corticosteroids. Clinical manifestation can include uveitis, episcleritis, keratopathy and dry eyes. In the most severe cases IBD leads to blindness [80]. Sclerosing cholangitis is the most frequent liver manifestation of IBD with prevalence of 1-4% in UC. It affects the bile ducts by causing strictures [80]. Furthermore, cutaneous manifestations such as erythema nodosum, pyoderma gangrenosum and oral ulcerations may

also occur. They alter the skin color and cause pain [80]. The chance for development of one or more of the extra-intestinal manifestations is 10-30% in patients with UC.

The development of IBD is still not completely understood, but there has been progress in the understanding of its pathology. In principal there are 3 important mechanisms in the development of IBD. First, defects in epithelial integrity/permeability allow bacteria to penetrate the colon. Second, defects in innate immune cells and third defects in cells of the adaptive immune system lead to altered reactions on bacteria and bacterial compounds [84].

Today it is accepted that a coaction of genetic and environmental factors including smoking, diet, drugs, geographical and social status, stress, microbial agents, intestinal permeability and appendectomy are involved in the development and course of IBD (Figure 4) [85,86]. As an environmental factor, cigarette smoke is known to protect against UC, but it increases the risk for CD [87,88]. It also ameliorates the course of disease in UC, but worsens its course in CD. Recently it has been shown that the underlying mechanism of protection in UC is the recruitment of invariant natural killer T (iNKT)-cells [89], however this leads to an increase in adenoma formation in the colon [90].

Genome-wide association studies led to the identification of genes that contribute to disease susceptibility. The first identified single nucleotide polymorphisms (SNPs) in CD were discovered in the nucleotide-binding oligomerization domain 2/caspase recruitment domain 15 (NOD2/CARD15) gene; also a frame-shift was identified in this gene [91,92]. NOD2/CARD15 is an intracellular ligand for muramyl dipeptide, a motif common to gram-positive and gram-negative bacterial cell walls [93]. The identification of this polymorphism was the first proof for the involvement of the innate immune system to CD since NOD2/CARD15 functions as a pattern recognition receptor (PRR) [94]. This led to the hypothesis that a dysregulated host response to luminal bacteria causes CD [95]. Many other genes were identified as risk factors for CD and UC such as the IL-23R gene and the autophagy-related 16-like 1 (ATG16L1) gene [95]. Interestingly, there seems to be a higher genetic influence on CD than on UC. This was shown in studies of twins where a stronger concordance with CD than with UC was apparent [81]. There are also mutations in autophagy factors, which are involved in restricting microbial growth within the host tissue [96], thus leading to reduced pathogen clearance and increased intracellular growth of bacteria [97]. Furthermore, there are SNPs in genes responsible for UC such as STAT3 or X-box binding protein 1 (XBP1) [98,99]. Taken together, risk loci for UC comprise genes responsible for dysfunction of the epithelial barrier, apoptosis and autophagy and defects in transcriptional regulation. Overlaps between UC and CD are present for instance for genes in the IL-23

signaling pathway like IL-23R, JAK2 and STAT3, of which the latter is also involved in IL-6 signaling [81].

Other factors involved in IBD and cancer development are mucin 2 (MUC2), junction adhesion molecule A (JAM-A), adenoma polyposis coli protein (APC) and β-Catenin [100]. Dysregulation or lack of these proteins contributes to development of IBD or adenoma formation in the intestine. MUC2 forms the mucin layer of the intestine which is important for separation of epithelial cells from commensal bacteria [101]. Mice lacking MUC2 develop colitis and colitis associated CRC spontaneously [100,102,103]. Ablation of JAM-A in mice results in enhanced permeability and inflammation in the colon [104]. Since JAM-A is part of tight-junctions and altered expression of tight junction proteins was reported for CRC, the role of an intact epithelial barrier in the colon for resistance against IBD is highlighted [105]. In the course of IBD, development of colitis associated CRC is speculated to be influenced and tumor progression is speculated to be increased by breakdown of barrier function. Early adenoma cells loose APC expression, which normally acts as a tumor suppressor and β-Catenin is activated, leading to proliferation and loss of differentiation of enterocytes [106]. This might cause loss of barrier function. As a consequence, increased numbers of commensal bacteria can penetrate the epithelium and are recognized by tumor associated macrophages. Macrophages produce IL-23, activating pro-inflammatory cells such as T helper 17 (T_H17) cells, which produce IL-17. IL-17 activates adenoma cells to phosphorylate STAT3 increasing tumor growth [100].

1.3.1 Mouse models of IBD

There are many different murine models for human IBD. In principle there are genetic models for IBD, models based on immune cell transfer and chemically induced ones [84]. As genetic models there are KO mice for N-cadherin, Keratin 8 and multiple drug resistant (MDR) gene 1. Even though the nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) pathway is one of the most prominent signaling pathways induced in inflammation, genetic blockade of NFκB leads to development of colitis [84]. Interestingly, mice lacking STAT3, a transcription factor, which is activated upon IL-6 stimulus, in macrophages and neutrophils, develop spontaneous enterocolitis [107]. Mice deficient for IL-10, an anti-inflammatory cytokine, develop chronic enterocolitis [108]. Senescence accelerated mouse (SAM)P1/Yakult Central Institute for Microbiological Research Tokyo (Yit) mice (SAMP1/Yit) develop a spontaneous ileitis which is similar to human CD [109,110]: In this mouse strain, STAT3 activation by IL-6 trans-signaling plays an important role for disease,

since administration of sgp130Fc ameliorates disease severity and blocks STAT3 activation [111]. Besides the mentioned genetic models for IBD, there are also others available.

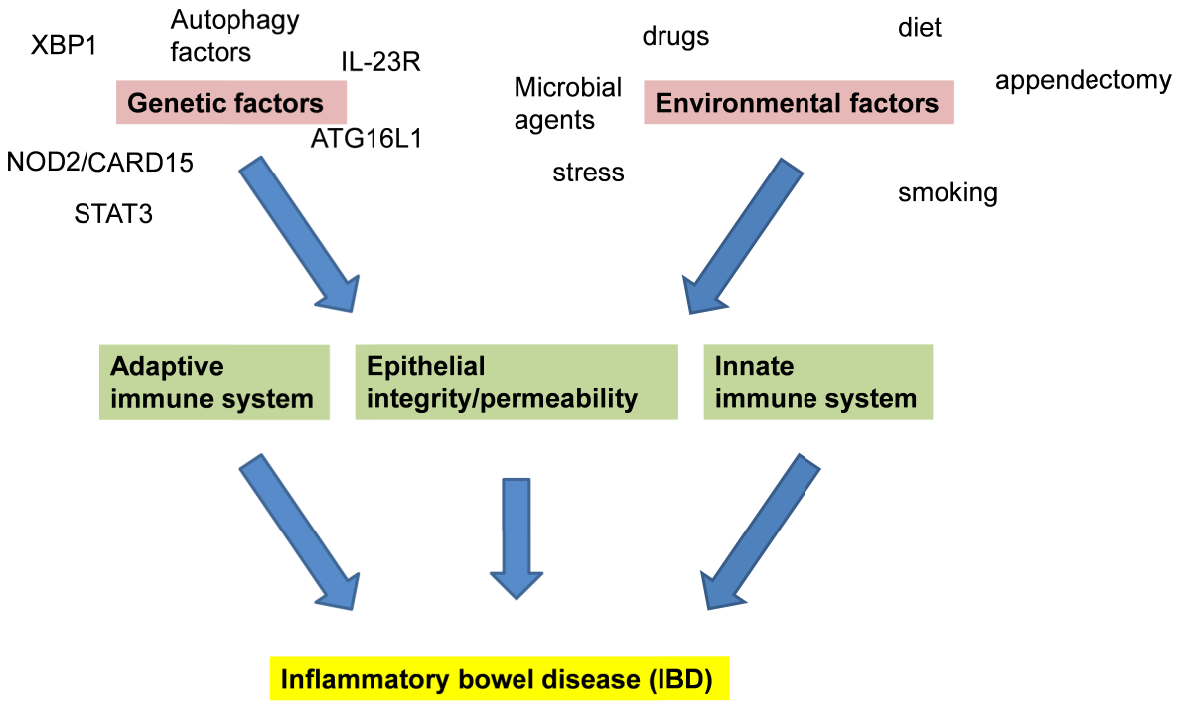

Figure 4: Factors influencing the development of IBD.
In principal there are 2 main factors leading to the development of IBD. On the one hand there are genetic factors like alterations in the IL-23R sequence or NOD2/CARD15 mutations [91,92,95]; on the other hand there are environmental factors like stress or smoking [86,89]. These groups influence the epithelial integrity/permeability or the immune system leading to IBD.

A characteristic which the immune cell transfer models have in common is that the transfer of subtypes of T cells leads to the development of colitis in T cell deficient mice. These mice can be rescued by the transfer of other subtypes of T cells or by breeding under germ-free conditions [84].

Chemical models for IBD are induced by application of trinitro benzene sulfonic acid (TNBS), oxazolone or dextran sodium sulfate (DSS). TNBS and oxazolone stimulate the adaptive immune system by haptenating autologous proteins or luminal antigens [112].

DSS is directly toxic to gut epithelial cells of the basal crypts and, therefore, affects the integrity of the mucosal barrier leading to an acute or pseudo-chronic colitis [112]. Another hypothesis for the action of DSS is destruction of the mucin coat in the colon, followed by necrosis of epithelial cells and neutrophil infiltration; in the end the crypt structure of the colon is lost [113]. After destruction of the epithelial barrier, DSS can be detected in many cells of the body like macrophages in the spleen, lymph nodes and copper cells in the liver

[113]. In this model, the innate immune system plays the main part, since severe combined immunodeficient (SCID) mice develop a DSS-induced colitis as well [114].
DSS is administered in drinking water of mice and induces an acute colitis with weight loss, bloody diarrhea, ulcerations and infiltration with granulocytes [113,115]. The susceptibility is mainly depending on the mouse strain used and the DSS (sulfatation rate, molecular weight, concentration) [113]. DSS-induced colitis can be performed using one cycle of DSS administration to the mice, eventually followed by a regeneration phase with pure tap water, this would lead to an acute DSS-induced colitis. Chronic DSS-induced colitis is caused repeating the DSS administration after the regeneration phase for several times, most common for 2 or 3 further cycles. Chronic DSS-induced colitis can also be modified by administration of azoxymethane (AOM), leading to development of CRC [116]. CRC is a long-term consequence in up to 30% of patients suffering from UC [83].
Taken together, if one wants to study the role of the adaptive immune system in colitis in a simple model, one can choose TNBS or oxazolone for inducing colitis, whereas the DSS model of colitis is the best choice for studying the role of the innate immune system in colitis. In the end, one has to consider the results of all models for getting as close as possible to an understanding of the mechanisms involved in colitis.

1.3.2 IL-6 and IL-6R are important for tissue regeneration

IL-6 and IL-6R are indispensable factors for healing processes. In the case of liver damage, trans-signaling seems to be more important for regeneration than classic signaling since administration of Hyper-IL-6 immediately after partial hepatectomy leads to a faster onset of regeneration in the remaining part of the liver [98]. Hyper-IL-6 is a fusion protein of IL-6 and the soluble IL-6 receptor that mimics IL-6 trans-signaling [117,118]. Furthermore, Hyper-IL-6 gene therapy but not IL-6 gene therapy reverses fulminant hepatic failure [25,119].
IL-6 is also important for wound healing [120]. Interestingly, this is only true for IL-6 itself, whereas the IL-6R plays a minor role in wound healing. This finding, shown by an IL-6 KO (*Il-6*$^{-/-}$) mouse, which had a delay in wound healing in contrast to an IL-6R KO (*Il-6r*$^{-/-}$) mouse showing no differences in wound closure compared to wt mice, remains obscure [121]. Interestingly, the double KO (*Il-6*$^{-/-}$/*Il-6r*$^{-/-}$) did not have a delay in wound closure, either; phenotypically only the *Il-6*$^{-/-}$ mouse was different from the other genotypes in respect to wound closure.

1.3.3 The good, the bad, the ugly role of IL-6 and IL-6R in inflammation

In general there are two types of inflammation, acute and chronic inflammation. In the beginning of acute inflammation, leukocytes mainly neutrophils infiltrate the destructed tissue [122]. Neutrophils have a short lifespan and undergo apoptosis after phagocytosis of microbes [123]. Given that apoptosis induces ADAM17 to shed the ectodomain of the IL-6R [39], the level of sIL-6R rises during inflammation [124]. The sIL-6R renders surrounding tissue cells susceptible to an IL-6 trans-signal leading to the expression of monocyte chemo-attractant protein-1 (MCP-1) [125]. This is necessary for the infiltration of monocytes into the inflamed tissue [126]. The monocytes than clear the apoptotic neutrophils [127]. Blockage of IL-6-signaling has little effects on the infiltration of neutrophils but impairs the infiltration of monocytes [39]. Here, IL-6 trans-signaling plays an important role for clearance of acute inflammation by monocytes.

A dysregulation of inflammatory mediators is found in IBD. Patients suffering from CD have increased IL-6 [128] as well as sIL-6R levels [129]. In a model for acute inflammation, the IL-6R is shed from neutrophils during apoptosis [39]. Shedding is also induced by acute phase protein C-reactive protein (CRP) in macrophages [130], by bacterial toxins [131] or by microbial metalloproteinases in human monocytes [132]. Another important source for sIL-6R is the liver [121]. The sIL-6R than forms a complex with IL-6 that is able to bind to a gp130 homodimer [133]. In IBD, immune cells such as neutrophils infiltrate in the colonic tissues, therefore they might be an important source for sIL-6R and thereby be important inducers of IL-6 trans-signaling. In models for acute inflammation, IL-6 trans-signaling is important for the recruitment of monocytes [39]. Given that monocytes in adenoma tissues increase tumor progression [100], trans-signaling in the colon might promote tumor growth.

In human IBD, T cells from intestinal tissues are very resistant to apoptosis [134]. This indicates a role for T cells in IBD. Possibly, trans-signaling on T cells which are initially also responsive to IL-6 alone leads to translocation of STAT3 into the nucleus and thereby expression of anti-apoptotic genes. Interestingly, T cells shed the IL-6R when they are activated, so that they become responsive to trans-signaling only in an ongoing inflammation [135]. The complex of IL-6/sIL-6R can also bind to epithelial cells leading to STAT3 translocation into the nucleus and expression of anti-apoptotic genes as well as to induction of cell proliferation.

IL-6 alone induces a T cell response which possibly worsens IBD, but in a complex with the sIL-6R, T cell response is sustained after shedding of the IL-6R from the T cells surface (Figure 5) [136]. Nevertheless, IL-6 KO mice display a higher inflammatory score than wt

animals, due to increased apoptosis and reduced cellular proliferation in some T cell independent studies [137]. Others show, as one might expect, that IL-6 KO mice are less susceptible to IBD compared to their littermate controls [138]. IL-6 is known to play an important role in many inflammatory disorders and blockade by an IL-6R antibody might be a good therapeutic approach in many of those [139]. Tocilizumab as an IL-6R antibody is already approved in the US, EU and Japan for the treatment of RA and other diseases. Blocking IL-6 trans-signaling was shown to ameliorate chronic inflammatory diseases [29], therefore blocking IL-6 trans-signaling by soluble variants of gp130 might offer an attractive alternative treatment option by leaving IL-6 classic signaling intact (Figure 1 D) [32]. An optimized variant of the trans-signaling inhibitor sgp130Fc is currently in clinical trials. To investigate whether the role of IL-6 in DSS-induced colitis is beneficial or detrimental, and therefore IL-6 blockade or IL-6 trans-signaling blockade might be a useful target in therapy for UC, we performed DSS-induced colitis in mice.

The role of STAT3 is still controversial: On one hand, activation of STAT3 in T cells leads to resistance of those cells against apoptosis. On the other hand, deletion of STAT3 in murine epithelial cells leads to the development of a fulminant form of enterocolitis [140] whereas mice with a genetic hyperactivation of STAT3 are resistant to colitis [141]. STAT3 activation turns out to be a double edged sword: in intestinal epithelial cells it prevents tissue damage during IBD at the expense of a higher risk for cancer development whereas hyperactivation of STAT3 in T cells leads to anti-apoptosis and thereby to the maintenance of inflammation.

A long-term consequence of IBD is CRC. In several studies, the role of IL-6 and STAT3 in inflammation associated CRC was analyzed. Depending on the setting, IL-6 KO mice develop stronger colitis, but fewer tumors than wt mice [137]. In contrast, mice with a hyperactivated form of STAT3 show an increased tumor incidence [141]. In Balb/c mice, STAT3 activation is increased during colitis associated premalignant cancer [143].

In a TNBS-induced colitis model, it was elucidated if blockade of IL-6 trans-signaling or trans- and classic signaling ameliorates IBD. An IL-6R antibody was used to block both, trans- and classic signaling, sgp130Fc to block trans-signaling only. It was shown, that both treatments lead to decreased inflammation compared to control mice [134]. Usage of an IL-6R antibody in patients suffering from CD treated inflammation [144].

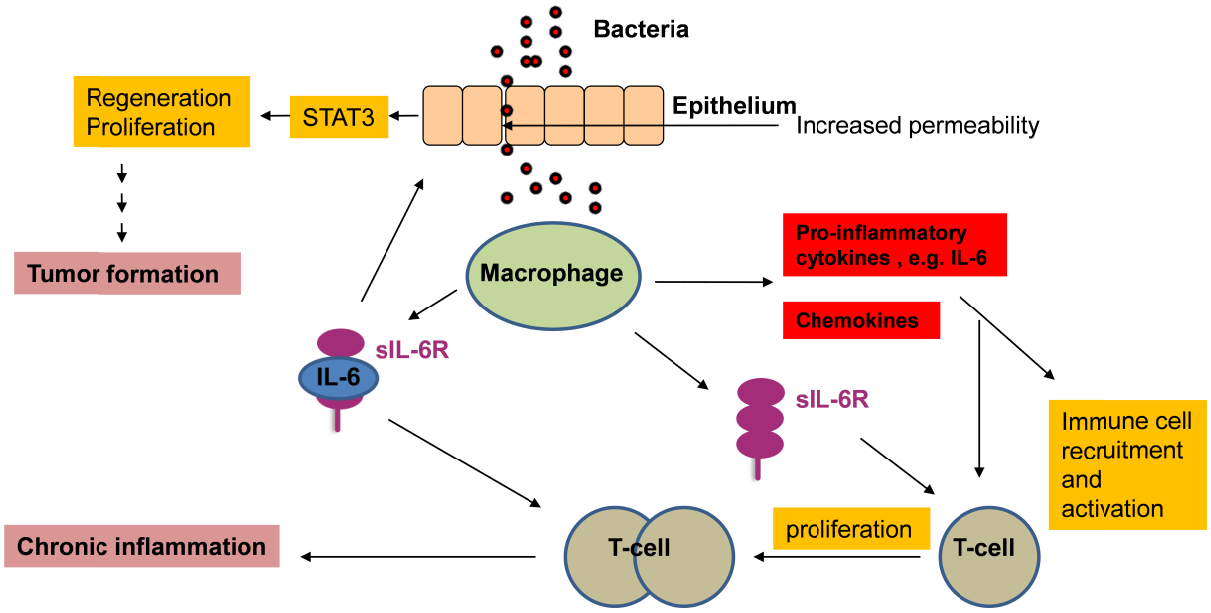

Figure 5: Cellular interactions during development of IBD.
The initial event in the development of IBD is an increased permeability of the intestinal epithelium allowing bacteria to penetrate from the intestinal lumen into the lamina muscularis and the submucosa. There they are recognized by macrophages which can normally limit bacterial burden without a strong inflammatory reaction. If macrophages cannot limit bacterial burden or show an excessed reaction, they produce large amounts of pro-inflammatory cytokines such as IL-6, as well as chemokines. Macrophages also shed their IL-6R, producing sIL-6R. Further immune cells like T cells are recruited. Trans-signaling on T cells leads to proliferation and in the end to chronic inflammation of the colon. The complex of IL-6/sIL-6R can also activate trans-signaling in the epithelium inducing STAT3 phosphorylation and thereby regeneration and proliferation of the intestinal tissues. As a long term-consequence, the interplay between tissue destruction and regeneration can end up in tumor formation [135,142].

These data highlight IL-6 as an important factor in IBD. Furthermore, IL-6 levels in serum of UC patients correlate with the chance of failure of intravenous corticosteroid therapy [145]. Colonic IL-6 levels are significantly upregulated in TNBS-induced UC in Wistar rats [146]. IL-6 levels in the colon of DSS treated mice are elevated and augment the infiltration of granulocytes and tissue damage via trans-signaling [147]. Interestingly in patients suffering from UC there was no increase in mRNA level of *IL-6* in mucosal tissues in contrast to patients suffering from CD [148]. On the other hand there were increased IL-6 levels in perfusion fluid from investigated rectal and sigmoid segments of UC patients [149]. This indicates that IL-6 is not the initial reason for the development of UC, but that immune cell infiltrates produce the colonic IL-6 which leads via trans-signaling by binding to the sIL-6R and membrane bound gp130 to the manifestation of UC. This hypothesis is supported by the finding that IL-6 levels in the serum of CD and UC patients return to normal levels after resection of inflamed bowel segments [150].

In the course of UC, there is a complex interplay between tissue damage and healing processes. IL-6 is also known to play an important role in healing processes. In chapter 5 of

this thesis, we demonstrate that the IL-6R neither plays a role in an acute DSS-induced colitis nor in a chronic DSS-induced colitis. This is consistent with the findings for wound healing [121]. We further investigated the role of IL-6 in an acute DSS-induced colitis using a neutralizing IL-6 antibody. Previous reports showed that IL-6 itself seems to have a role in this model for UC, but it remained uncertain if it was a beneficial or a detrimental role [137,138]. Giving high doses of DSS resulted in an increased mortality in *Il-6*$^{+/+}$ compared to *Il-6*$^{-/-}$ mice [138]. In contrast to this result, administration of a low dose of DSS was accompanied with an increased regeneration of *Il-6*$^{+/+}$ compared to *Il-6*$^{-/-}$ mice, but with higher tumor formation as a long term consequence [137]. In our study we used low amounts of DSS to mimic the latter experiment, but, surprisingly we found significant results similar to the first referred study [138]. Therefore we conclude that the IL-6R does not play a role in DSS-induced colitis whereas IL-6 has a detrimental effect in an acute DSS-induced colitis and its blockade might serve as a therapy for patients suffering from UC.

2 AIMS AND OBJECTIVES

Autoimmune diseases are characterized by an immune system directed against its own body. As a long-term consequence of the sustained destruction and regeneration processes, cancer is common in some autoimmune diseases such as UC. In UC, but also in RA and others, IL-6 is known to play a role. The aim of this doctoral thesis is to investigate mechanisms to block IL-6 signaling and to further elucidate the role of IL-6, IL-6R and gp130, all involved in IL-6 signaling, for autoimmune diseases.

The first aim comprises of investigations concerning the β receptor of IL-6, gp130. Gp130 is the main signal transducing receptor of the IL-6 family of cytokines. Soluble isoforms of gp130 are naturally occurring, but also recombinantly expressed for blocking IL-6 trans-signaling in patients suffering from different autoimmune diseases. In this doctoral thesis, a novel variant of gp130 is investigated after recombinant expression of the proteins for inhibitory properties on IL-6 trans-signaling. It is further analyzed if the novel variant is evolutionary conserved. Besides dominant-negative variants of gp130, gp130 is also known to be mutated in IHCA. This thesis addresses the question about consequences on cellular homeostasis caused by a frequently occurring in-frame deletion in gp130 and if a potential therapy of IHCA could be blockade of the constitutively active gp130 variant.

The second aim is to resolve the role of IL-6 and its α-receptor the IL-6R in a mouse model for human UC. IL-6 is known to exhibit pro-inflammatory as well as anti-inflammatory properties. As its anti-inflammatory property, regeneration processes are promoted by IL-6 signaling. In liver regeneration, IL-6 trans-signaling is crucial, in wound healing lack of IL-6 leads to decreased re-epithelialization and wound closure, whereas the IL-6R is optional for the latter one. In some autoimmune diseases IL-6 levels are increased, emphasizing its pro-inflammatory properties. Thus, this thesis is dedicated to the investigation of the role of the IL-6R in DSS-induced colitis in mice. It furthermore addresses the question, which role IL-6 plays in this model for human UC. Both results should answer the question, whether IL-6 and the IL-6R are promising targets in human UC.

CHAPTER 1

An interleukin-6 receptor-dependent molecular switch mediates signal transduction of the IL-27 cytokine subunit p28 (IL-30) via a gp130 protein receptor homodimer.

Published in: Journal of Biological Chemistry

Impact factor: 4.651

Own Proportion

To this work: 5 %

Establishment of mice strains

Genotyping of mice

Sacrifice of mice for spleen isolation

Garbers, C, Spudy, B, Aparicio-Siegmund, S, Waetzig, GH, Sommer, J, Hölscher, C, Rose-John, S, Grötzinger, J, Lorenzen, I, and Scheller, J (2013) An interleukin-6 receptor-dependent molecular switch mediates signal transduction of the IL-27 cytokine subunit p28 (IL-30) via a gp130 protein receptor homodimer. *The Journal of biological chemistry* **288**: 4346-4354.

CHAPTER 2

Constitutively Active Mutant gp130 Receptor Protein from Inflammatory Hepatocellular Adenoma Is Inhibited by an Anti-gp130 Antibody That Specifically Neutralizes Interleukin 11 Signaling

Published in: Journal of Biological Chemistry

Impact factor: 4.651

Own Proportion

To this work: 45 %

Cloning and transfection of C-terminal tagged gp130 variants

Transduction of C-terminal tagged variants into Ba/F3-gp130 cells

Selection of transduced Ba/F3-gp130 cells

Proliferation Assays

Western Blotting

Co-precipitation studies

Inhibition studies using B-P4 and B-R3 gp130 antibodies

Sommer, J, Effenberger, T, Volpi, E, Waetzig, GH, Bernhardt, M, Suthaus, J, Garbers, C, Rose-John, S, Floss, DM, and Scheller, J (2012) Constitutively active mutant gp130 receptor protein from inflammatory hepatocellular adenoma is inhibited by an anti-gp130 antibody that specifically neutralizes interleukin 11 signaling. *The Journal of biological chemistry* **287**: 13743-13751.

CHAPTER 3

Alternative Intronic Polyadenylation generates the Interleukin-6 trans-signaling Inhibitor sgp130-E10

Published in: Journal of Biological Chemistry (under revision)

Impact factor: 4.651

Own Proportion

To this work: 90 %

Transfection and selection of cells

Protein expression

Protein purification

Analysis of purified protein and functional analysis

Alignments

Isolation of PBMCs

PCR and qPCR analysis

Writing of the manuscript

Sommer, J, Garbers, C, Wolf, J, Trad, A, Moll, JM, Sack, M, Fischer, R, Grötzinger, J, Waetzig, GH, Floss, DM, and Scheller, J (2014) Alternative intronic polyadenylation generates the interleukin-6 trans-signaling inhibitor SGP130-E10. *The Journal of biological chemistry*, Epub ahead of print.

CHAPTER 4

Inhibition of Protein Kinase II (CK2) prevents induced Signal Transducer and Activator of Transcription (STAT) 1/3 and constitutive STAT3 activation

Published in: Oncotarget

Impact factor: 6.636

Own Proportion

To this work: 5 %

Cloning and transfection of C-terminal tagged gp130 variants

Transduction of C-terminal tagged variants into Ba/F3-gp130 cells

Selection of transduced Ba/F3-gp130 cells

Aparicio-Siegmund, S, Sommer, J, Monhasery, N, Schwanbeck, R, Keil, E, Finkenstädt, D, Pfeffer, K, Rose-John, S, Scheller, J, Garbers, C (2014) Inhibition of Protein Kinase II (CK2) prevents induced Signal Transducer and Activator of Transcription (STAT) 1/3 and constitutive STAT3 activation. *Oncotarget* **5**: 2131-2148.

CHAPTER 5

Interleukin-6 but not the Interleukin-6 receptor plays a role in Dextran Sodium Sulfate induced Colitis

Published in: International journal of molecular medicine (submitted)

Impact factor 1.957

Own Proportion

To this work: 95 %

Establishment of mouse strains

Genotyping of mice

Phenotypic analysis of mice

Experimental colitis

Analysis of mice samples after the colitis

Writing of the manuscript

Sommer J., Engelowski E., Baran P., Garbers C., Floss DM. and Scheller J. (2014) Interleukin-6 but not Interleukin-6 receptor plays a role in recovery from Dextran Sodium Sulfate-induced Colitis. *International journal of molecular medicine*, Epub ahead of print.

3 GENERAL DISCUSSION

This doctoral thesis deals with the functional analysis of the IL-6 family of cytokines. The family shares the common beta-receptor gp130. In the first 4 chapters, gp130 is in the focus of analysis. First of all, IL-30 is characterized as a novel member of the IL-6 family of cytokines that can signal either in a complex with the IL-6R at low concentrations or without any binding partner at high concentrations via a gp130 homodimer. Furthermore, a frequently occurring somatic gp130 deletion variant in inflammatory hepatocellular adenomas is analyzed. Ba/F3-gp130 cells were transduced with the gp130ΔYY variant. Cells started to proliferate in the absence of any cytokine and showed a constitutive STAT3 activation. Interestingly, these processes could be inhibited by a gp130 antibody specifically neutralizing IL-11 signaling. Besides membrane bound gp130, there are also soluble isoforms harboring antagonistic properties for IL-6 trans-signaling. I identified a novel sgp130 variant, termed sgp130-E10, originating from alternative IPA. To the best of my knowledge, this was the first report on alternative IPA for a receptor with associated kinases. We fused sgp130-E10 to a human Fc tag or to a myc-His tag and showed that the purified protein inhibits STAT3 phosphorylation and trans-signaling dependent proliferation of Ba/F3-gp130 cells. Furthermore, it was shown that protein kinase II (CK2) is indispensable for gp130 mediated signal transduction. Inhibition of CK2 results in loss of gp130 activation not only in cytokine dependent signaling but also in the constitutively active gp130 variant gp130ΔYY.

In the final chapter of this thesis, it is shown that the IL-6R is not involved in pathogenesis of a murine DSS-induced colitis model, in contrast to IL-6. Tissue specific and complete IL-6R deficient mice are bred and challenged in a DSS-induced colitis model. Neither the complete KO, nor the tissue specific KO mice had a different susceptibility in this model of disease compared to wt animals. Neutralizing IL-6 in wt mice leads to a decreased susceptibility to DSS-induced colitis, indicating a detrimental role for IL-6 in this model of disease.

3.1 P28 HAS AGONISTIC, BUT NO ANTAGONISTIC PROPERTIES

P28 binds to Epstein-Barr virus induced protein 3 (EBI3) forming the cytokine IL-27 [151]. IL-27 induces signals via a heterodimer of WSX1/gp130 (Figure 6A) [7]. IL-27 has anti-inflammatory properties [152]. In the absence of EBI3, p28 as a monomer can also induce signal transduction as the cytokine IL-30 [8,153]. IL-30 induces signal transduction in IL-27 responsive cells activating STAT1 and STAT3 [8]. In contrast to this, in other studies IL-30 did not induce STAT phosphorylation, but inhibited IL-6 and IL-27 signaling in responsive cells [153]. In the first chapter of this thesis, it is shown that IL-30 forms a complex with the membrane bound and the soluble IL-6R [33]. In contrast to p28/EBI3 recruiting a heterodimer of WSX1/gp130 [7], this complex recruits a homodimer of gp130 for IL-30 classic or trans-signaling (Figure 6B, C), respectively [33]. Interestingly, the p28/(s)IL-6R complex did not interfere neither with IL-6 nor with IL-27 signaling, thus harboring agonistic, but no antagonistic properties [33].

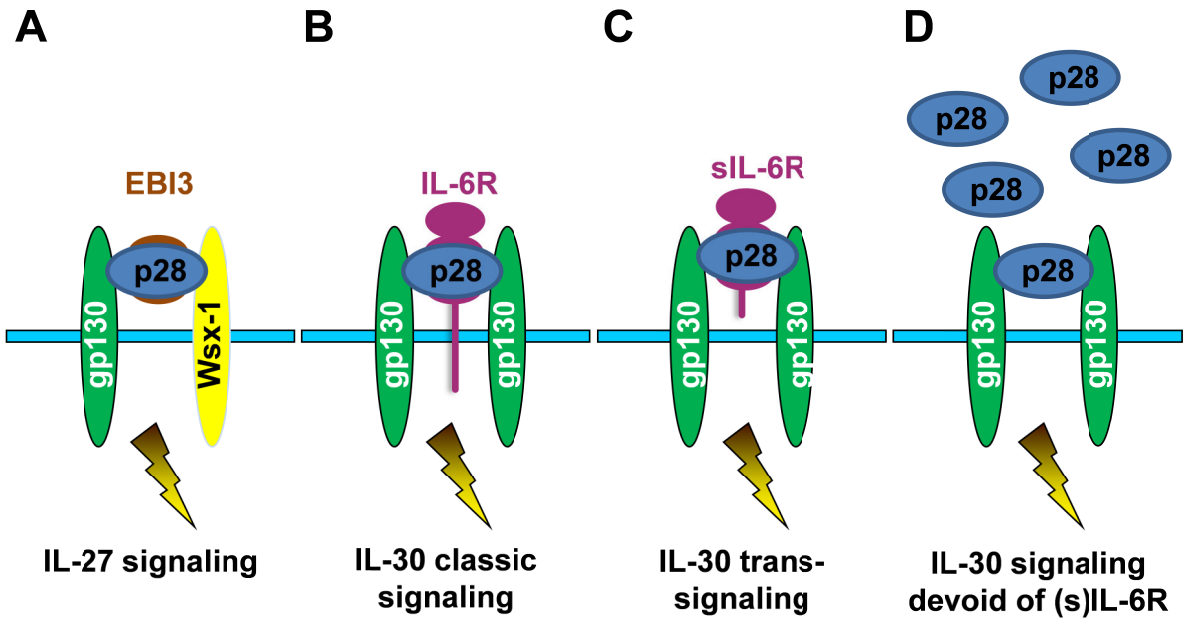

Figure 6: Signaling of p28.
A) EBI3 binds to p28 forming a complex termed IL-27, that subsequently recruits a heterodimer of gp130/Wsx-1 for signal transduction [7]. **B/C)** p28 (IL-30) also forms a complex with the membrane bound or soluble IL-6R recruiting a gp130 homodimer for signaling [8,33]. These processes resemble very much those of IL-6 classic and trans-signaling and can therefore be termed IL-30 classic and trans-signaling, respectively. **D)** In high concentration, p28 directly recruits and binds to a homodimer of gp130 mediating signals [33].

Furthermore, it is shown that IL-30 can induce signal transduction via gp130 even in the absence of membrane bound or soluble IL-6 receptor and EBI3, directly binding to a gp130 homodimer, but with a low affinity (Figure 6D) [33]. The low affinity of IL-30 to gp130 is evidence that IL-30 cannot interfere with IL-6 signaling, therefore IL-30 is unlikely to harbor antagonistic properties [33].

3.2 LIGAND-INDEPENDENT, IL-11-LIKE SIGNALING VIA GP130 RENDERS GP130 ONCOGENIC

Upon gp130 signaling, STAT3 gets activated [154]. STAT3 is a transcription factor involved in many human neoplastic malignancies such as multiple myeloma and a variety of cancers [60,155-158]. Constitutively activated STAT3 has oncogenic potential and is therefore designated as an oncogene [159,160]. Increased STAT3 phosphorylation is found in IHCAs, resulting in 60% of IHCAs from somatic in-frame deletions in gp130 [59]. Therefore, gp130 can be considered as a proto-oncogene. In chapter 2 of this thesis, we investigated one of the frequently occurring in-frame deletion variants of gp130, gp130ΔYY. IL-6 trans-signaling dependent Ba/F3-gp130 cells were transduced with the gp130ΔYY variant. The Ba/F3-gp130-gp130ΔYY showed ligand-independent proliferation and constitutive STAT3 phosphorylation [61]. Interestingly, STAT3 phosphorylation and cellular proliferation in the absence of Hyper-IL-6 was lost for the gp130ΔYY variant lacking domain 1 [61]. Domain 1 is crucial for the activation of gp130 homodimers by IL-6 and IL-11 [161], indicating that the gp130ΔYY variant has a similar mechanism of activation as gp130 upon IL-6 and IL-11 stimulus. However, the neutralizing gp130 antibody B-T2 failed to inhibit constitutively active gp130 STAT3 phosphorylation [61]. B-T2 is directed against domain 1 of gp130 and specifically inhibits IL-6, but not IL-11 signaling [162]. This indicates that the activation mechanism is more similar to IL-11 signaling than to IL-6 signaling from which it seems to be distinct. Moreover, we could show that the neutralizing gp130 antibody B-P4, which specifically inhibits IL-11 signaling (Figure 7C), also inhibits STAT3 phosphorylation in Ba/F3-gp130-gp130ΔYY cells without interfering with Hyper-IL-6 dependent STAT3 phosphorylation (Figure 7D) [61,162].

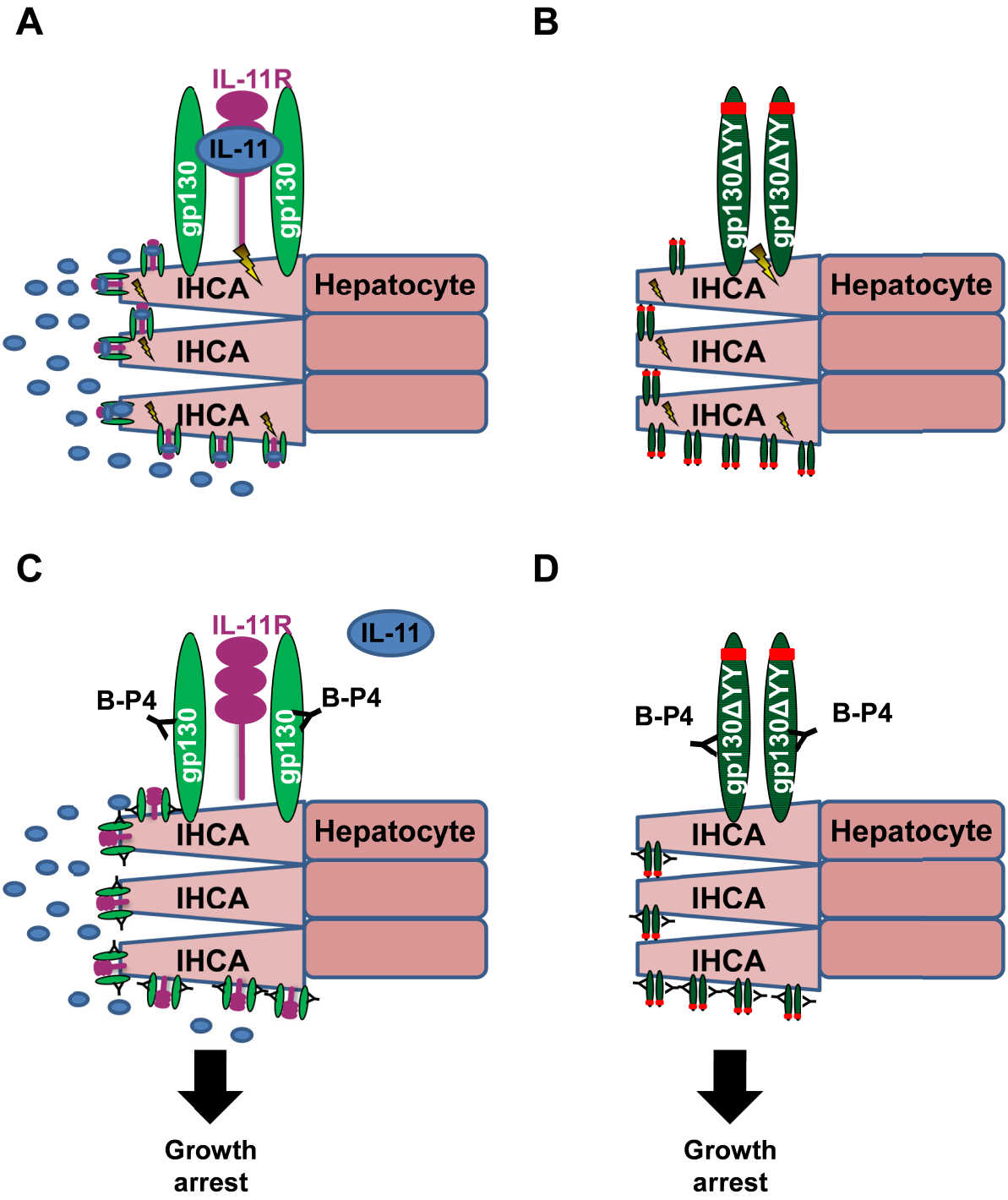

Figure 7: Loss of IL-11 dependence in IHCAs by gp130 in-frame deletion and blockade of IL-11 and IL-11 like signaling in IHCAs.
A) IL-11 is overexpressed in IHCAs [59]. Proliferation of IHCA cells depends on IL-11 signaling. **B)** The deletion variant gp130ΔYY mimics IL-11 signaling in the absence of any ligand [61]. IHCAs become independent of IL-11. **C/D**). The IL-11 signaling neutralizing gp130 antibody B-P4 inhibits IL-11 signaling and the constitutively active gp130 variant gp130ΔYY [61]. Inhibition of IL-11 signaling and IL-11 like signaling might lead to growth arrest of IHCAs.

IL-11 was shown to promote gastric cancer inducing STAT3 phosphorylation [163]. IL-11 is also overexpressed in IHCAs indicating that IHCAs are either dependent on IL-11 signaling (Figure 7A) or IL-11 like signaling via a constitutively active gp130 variant mimicking IL-11 signaling (Figure 7B) [59]. Interestingly, IL-11 was only overexpressed in IHCAs that did not harbor gp130 mutations [59]. Thus IL-11 overexpression might be an early event in IHCAs until they overcome necessity of IL-11 by somatic in-frame deletions in gp130 mimicking IL-11 signaling.

B-P4 administration might be used as a therapy for IHCAs both in the early phase where adenomas are dependent on IL-11 and also in the later stage when they have overcome their dependence on IL-11 by gp130 in-frame deletions rendering gp130 constitutively active.

3.3 SGP130-E10: A CONSERVED GP130 ISOFORM HARBORING DOMINANT-NEGATIVE PROPERTIES

Soluble gp130 isoforms compete with the membrane bound receptor for the binding to the complex of IL-6/sIL-6R, thereby inhibiting IL-6 trans-signaling [66]. In human serum, levels of about 400 ng/ml of sgp130 variants are found [67]. These variants have a molecular weight of 50, 90 and 110 kDa, respectively [67-69]. The smallest variant originates from differential splicing and is termed sgp130-RAPS [68]. sgp130-RAPS inhibits IL-6 trans-signaling. The other two proteins are of unknown origin. There are two additional differentially spliced variants of gp130 resulting in soluble isoforms, but they have never been proven to exist on protein level [70,71].

Besides differential splicing, alternative IPA sites have been identified in many genes [75]. Usage of an intronic PAS leads to shortened mature mRNAs and thereby to truncated protein isoforms [76]. If the C-terminal part of a full-length protein is important for its function, intronic polyadenylation might abolish or alter the function of the shortened protein isoform [76]. Recently, soluble dominant-negative receptor-tyrosine kinase isoforms have been identified. These isoforms originated from alternative IPA [76].

In chapter 3 of this thesis, we describe a novel sgp130 mRNA which results from alternative IPA in intron 10. The resulting isoform (sgp130-E10) lacks domain 5 and 6, the transmembrane and the intracellular domain. Sgp130-E10 is mainly expressed in PBMCs and has dominant-negative properties in IL-6 trans-signaling. Recombinant sgp130-E10Fc inhibited proliferation of IL-6 trans-signaling dependent Ba/F3-gp130 cells and reduced STAT3 phosphorylation in those cells upon Hyper-IL-6 stimulus.

3.3.1 Sgp130-E10 is conserved in many mammals - indication for its relevance

IL-6 and the sIL-6R are elevated in a variety of autoimmune diseases [164,165]. IL-6 binds to the sIL-6R forming a complex that triggers IL-6 trans-signaling via a gp130 homodimer [5,166]. IL-6 trans-signaling is, in contrast to IL-6 classic signaling, important for the maintenance of the inflammatory state [166]. In human, IL-6 and sIL-6R levels in the serum are elevated in RA and IBD [167-170]. In rodent models for human IBD such as DSS-induced colitis in mice and TNBS-induced colitis in rats, animals have increased levels of IL-6 in the serum [146,147]. Dogs suffering from a form of canine IBD have elevated IL-6 levels in the serum and elevated mRNA levels of *IL-6* in the inflamed tissue sections [171]. Cats with a feline form of IBD have an increased copy number of *IL-6* transcripts compared to control animals [172]. Even though there are no reports investigating the role of sIL-6R in cats and dogs, one can estimate that IL-6 trans-signaling might be the important role of IL-6 in canine and feline IBD. This estimation is supported by Lee *et. al.* [147] who showed that blockage of trans-signaling in mice reduced disease severity [147]. Soluble gp130 variants are natural occurring inhibitors of IL-6 trans-signaling, but do not interfere with IL-6 classic signaling [68]. In RA, the amount of neutralizing antibodies directed against sgp130-RAPS correlates with disease severity [68]. This indicates that sgp130 isoforms play an important role in regulation of IL-6 trans-signaling.

IL-6 trans-signaling is a conserved signaling pathway in mammals [173-178]. Increased IL-6 levels are associated with inflammatory diseases among mammals [128,143,149,178]. In rat, mice and human, trans-signaling has been functionally associated autoimmune diseases [68,147,178]. In dog and cat IL-6 trans-signaling is likely the important driver of inflammation. Therefore, soluble gp130 variants might be important regulators of inflammation, not only in human, but also in other mammals. In chapter 3 of this thesis,we investigated whether the novel sgp130 variant, sgp130-E10, formed by alternative IPA is also present in other mammals. Conservation of genes is evidence for its importance. Therefore, we aligned the sequence of intron 10 which contains an alternative stop codon and the alternative intronic PAS of human to other mammals. Interestingly, we found that all mammals investigated have a conserved alternative stop codon and at least one alternative polyadenylation site within intron 10, except for rodents. This indicates that sgp130-E10 plays a role in those mammals. We also proved for dog and pig, that the mRNA for sgp130-E10 is present in PBMCs. Unfortunately, there is no antibody available specific for sgp130-E10 of dog and pig to confirm the results on protein level. Interestingly, intron 10 in dogs contains 5 alternative PAS. Therefore, clarifying the amount of sgp130-E10 in comparison to gp130

would be interesting. Furthermore, a transgenic mouse harboring an alternative stop codon and an alternative PAS in intron 10 would be worthwhile to create and challenge in models for acute inflammation and autoimmune diseases.

3.3.2 Sgp130-E10 as a natural trans-signaling inhibitor in inflamed tissues

As described above, sgp130 variants inhibit IL-6 trans-signaling while leaving IL-6 classic signaling intact [68,72]. Hyper-activation of gp130 by IL-6 trans-signaling is observed in many inflammatory disorders [166]. IL-6 trans-signaling is crucial for the development and maintenance of RA [134,179]. Blocking IL-6 signaling by a neutralizing IL-6R antibody and specific blockade of IL-6 trans-signaling by sgp130Fc are therapeutic approaches for the treatment of RA and other inflammatory disorders [180-183]. Three soluble isoforms of gp130 have been found in human serum [68,69]. One of these isoforms, sgp130-RAPS, which inhibits IL-6 trans-signaling, is neutralized by autoantibodies in RA [68]. This highlights the importance of soluble gp130 isoforms for limiting IL-6 trans-signaling to prevent an exaggerated immune response. The degree of loss of this regulation, indicated by the amount of autoantibodies against sgp130-RAPS in RA, positively correlates with disease severity [68].

We showed that sgp130-E10 is mainly expressed by PBMCs, such as monocytes and T cells. In an acute inflammation, neutrophils are the first cells recruited to the inflammatory site and limit bacterial burden (Figure 8A, B). Apoptosis of neutrophils is a natural stimulus of IL-6R shedding (Figure 8C) [39]. Subsequently, trans-signaling of the complex of IL-6 and the shed sIL-6R recruits monocytes/macrophages [39], which are essential for the resolution of inflammation. Sustained trans-signaling is associated with inflammatory disorders [135]. T cell receptor activation on T-helper cells induces shedding of the IL-6R [184]. T cells lose the sensitivity for IL-6 classic signaling and become responsive to IL-6 trans-signaling only. Due to STAT3 activation by IL-6 trans-signaling they become resistant against apoptosis [135,142]. Given that sgp130-E10 inhibits IL-6 trans-signaling and that PBMCs produce the highest amounts of sgp130-E10, one can speculate that sgp130-E10 serves as a natural limiter of IL-6 trans-signaling at the site of inflammation. In an acute inflammation, IL-6 trans-signaling is important to recruit monocytes for resolution of inflammation (Figure 8D) [39]. Neutrophils, which are the first cells at the site of inflammation, do not produce high amounts of sgp130-E10, thus, trans-signaling is not inhibited and monocytes are recruited (Figure 8C). At a later stage of inflammation, immune response has to be reduced. Monocytes might therefore produce high amounts of sgp130-E10 in order to limit their own recruitment to the

site of inflammation (Figure 8E). The immune reaction is accomplished (Figure 8F). It has to be mentioned, that the experiment for acute inflammation has been performed using an air-pouch model in mice [39]. Mice do not express sgp130-E10. Therefore, there must be additional mechanisms for limiting acute inflammation. Nevertheless, sgp130-E10 expression of PBMCs at the site of inflammation might contribute to limitation of inflammation, even though it is not crucial under normal conditions.

T cells, which are involved in some inflammatory diseases, also produce high amounts of sgp130-E10. Given that trans-signaling on T cells inhibits T cell apoptosis [135,142] production of sgp130-E10 at the site of inflammation might be an important self-regulatory step to keep self-tolerance at the periphery. Lack of sgp130-E10 production by T cells could be involved in the development of T cell dependent autoimmune diseases.

Even though our study did not show that sgp130-E10 is present in human serum, sgp130-E10 might play an important role in regulation of inflammation directly at the site of inflammation. Global expression and thereby inhibition of trans-signaling at sites where trans-signaling is needed, such as liver regeneration [25,119] or the early phase of inflammation [39], might not take place. A direct expression of sgp130-E10 by cells which are involved at a later time point of inflammation might be a mechanism of limiting the inhibition of trans-signaling to situations where an exaggerated trans-signaling leads to loss of self-tolerance.

The short half-life of sgp130-E10 might also be important to limit the inhibition of IL-6 trans-signaling. After resolution of inflammation, it is important that in the case of another infection a normal immune reaction can take place. Therefore, fast clearance of sgp130-E10 might be beneficial.

3.3.3 Short half-life of sgp130-E10 as a chance for therapy?

IL-6 trans-signaling is involved in several inflammatory disorders [147,166]. Therefore blockage of IL-6 trans-signaling is a drug target [28]. IL-6 trans-signaling can be blocked using an IL-6R antibody or sgp130Fc [29,180-183]. The IL-6R antibody tocilizumab is approved in the US, Japan and Europe for treatment of RA [180,181]. Usage of an IL-6R antibody does not only block IL-6 trans-signaling but also classic signaling since it binds to the soluble and to the membrane bound IL-6R [29]. Classic signaling is involved in many physiological processes such as B-cell differentiation and production of acute phase proteins in the liver [19,20,23]. Blockage of trans-signaling while leaving classic signaling intact might be favorable. Soluble gp130 variants such as sgp130Fc as a recombinant protein and sgp130-RAPS as a naturally occurring soluble isoform of gp130 specifically inhibit IL-6

trans-signaling [29,68]. The novel sgp130-E10 protein is a naturally occurring isoform of sgp130 harboring a short half-life and a low inhibitory potential compared to sgp130Fc. Its recombinant, Fc tagged form sgp130-E10Fc could be used as an alternative for or in combination with sgp130Fc.

In a murine model of sepsis, mice receiving sgp130Fc 24 h after challenge showed an increased survival of 80% compared to 45% without sgp130Fc injection, injection 24 h before challenge lead to a survival of 100% [185]. Neutralizing IL-6 and thereby blockade of IL-6 classic and trans-signaling did not have a comparable effect [185]. This highlights trans-signaling as the favorable target in sepsis. To prevent a septic shock, a fast and early blockage of IL-6 trans-signaling is important. Therefore sgp130-E10Fc might be interesting as a drug. Injection of sgp130-E10Fc would lead to blockage of IL-6 trans-signaling while leaving IL-6 classic signaling intact. The short half-life of sgp130-E10Fc guarantees blockade of trans-signaling when it is needed and a fast degradation afterwards to restore trans-signaling for liver regeneration and acute inflammation. Therefore, drug potential of sgp130-E10Fc should be checked in a mouse model of sepsis.

It might also be interesting to boost sgp130-E10 production by PBMCs. Alternative IPA is increased upon blockage of the normal splicing mechanism blocking small nuclear (sn) ribonucleic particle (RNP) U1 [76]. Therefore it should be clarified whether the induction of endogenous sgp130-E10 expression is possible. Induction of its expression directly at the site of inflammation would leave trans-signaling at other compartments of the body intact. This might be performed easily on the skin.

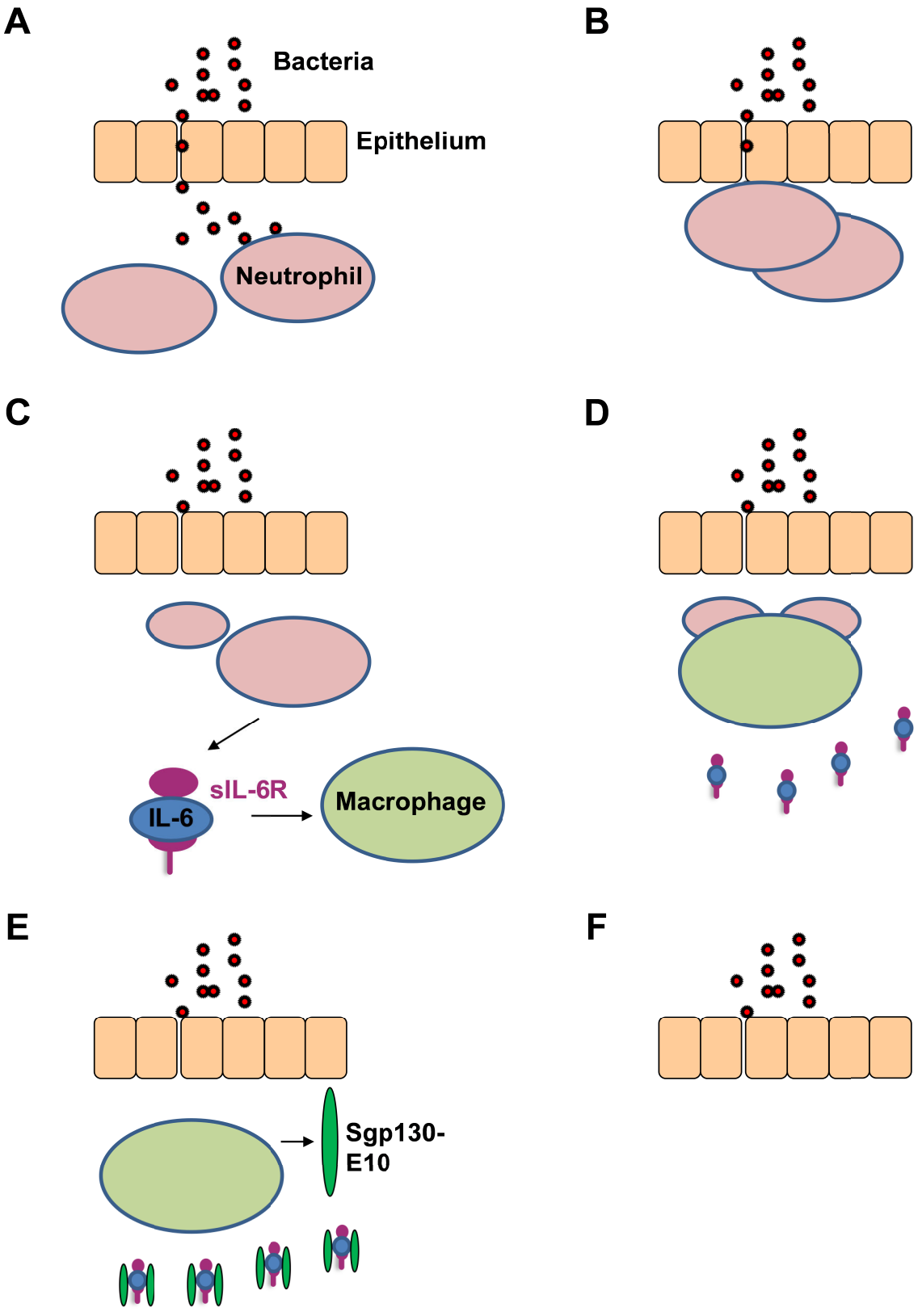

Figure 8: Sgp130-E10 might be important to resolve an acute immune response and to keep self-tolerance at the periphery.
A) Bacteria penetrate the epithelium. **B)**. Neutrophils phagocyte bacteria. **C)** Apoptosis of neutrophils leads to shedding of the IL-6R forming a complex with IL-6. Trans-signaling recruits monocytes/macrophages to the inflammatory site [39]. **D)** Macrophages clear apoptotic neutrophils [39]. **E)** Macrophages produce sgp130-E10 at the inflammatory site. Sgp130-E10 binds to the IL-6/sIL-6R complex to limit IL-6 trans-signaling and stop recruitment of further monocytes. **F)** Infection is limited, immune reaction is accomplished.

3.4 CK2 IS INDISPENSABLE FOR GP130 MEDIATED SIGNALING

CK2 is a ubiquitously expressed serine/threonine kinase phosphorylating more than 300 substrates [47,48]. It is formed by two catalytic α- and two regulatory β-subunits. KO of both results in embryonic lethality in mice [49-51]. A dysregulation of CK2 leads to development of several hematopoietic tumors and their progression, rendering CK2 inhibitors promising drugs [186-188]. One mechanism of tumor formation and progression in multiple myeloma is prevention of endoplasmic reticulum (ER) stress induced apoptosis by CK2 [189]. In chapter 4 of this thesis, we demonstrate that CK2 is involved in every type of gp130 mediated signal transduction. Furthermore, we show that JAK1 is the Janus kinase involved in gp130 mediated signaling, but not JAK2 and that JAK1 is dependent on CK2 activity. This is not only true for ligand dependent gp130 signaling, but also for constitutively active gp130 variants occurring in IHCAs. IL-6 and IL-11 are members of the IL-6 family of cytokines, involved in a variety of tumors such as colitis associated cancer or gastric tumors [128,163,190-192]. Blockage of signaling in these tumors might be a promising drug target. This study elucidates CK2 inhibition as a potential mechanism for blocking IL-6 family signaling and moreover also for blocking constitutively active gp130 variants.

3.5 IL-6 SIGNALING IN INFLAMMATORY BOWEL DISEASE

IL-6 is an important factor for several physiological processes such as B-cell differentiation, liver regeneration and wound healing [19,20,23,25,119,121,193]. Besides these positive properties, IL-6 is also involved in several inflammatory disorders such as RA and IBD [142,166,179]. Therefore, Tocilizumab, a neutralizing IL-6R antibody, is approved for treatment of RA in the US, Japan and Europe [180,181]. This antibody binds to the sIL-6R as well as to the membrane bound IL-6R, inhibiting trans- and classic IL-6 signaling [29]. Even though there are reports, that IL-6 classic signaling is sufficient to induce autoimmune diseases and trans-signaling can only sustain the inflammatory status [136], IL-6 trans-signaling is the detrimental kind of IL-6 signaling [28], since in autoimmune disease patients, the disease is already established and just needs to be sustained for progression. Most of the beneficial effects of IL-6 signaling, except for liver regeneration [25,119], are accomplished by IL-6 classic signaling [19,20,23]. Therefore blockage of IL-6 classic signaling is only a therapeutic possibility in severe cases of inflammatory disorders to avoid side-effects. When a patient is already suffering from autoimmune diseases such as CD or UC trans-signaling can

sustain the inflammatory state. Therefore, specific blockage of IL-6 trans-signaling without affecting classic signaling might be a good alternative for treatment with IL-6R antibodies [29]. Soluble variants of gp130 can specifically inhibit IL-6 trans-signaling [29]. Sgp130Fc, a fusion protein of sgp130 and the Fc part of a human IgG antibody is currently in clinical trials [194].

Despite the positive effects of blockade of IL-6 trans-signaling, only little is known about the precise role of IL-6 in IBD so far. There are many hints, that IL-6 plays a detrimental role in IBD. IL-6 levels in serum of UC patients correlate with the chance of failure of intravenous corticosteroid therapy [145]. This indicates that IL-6 is somehow important for resistance of the inflammation against anti-inflammatory drugs and possibly also against anti-inflammatory cytokines. In TNBS-induced UC in Wistar rats, colonic IL-6 levels are significantly increased [146]. The same is true for DSS treated mice, which causes granulocyte infiltration and tissue damage via trans-signaling [147]. Administration of sgp130Fc decreased disease activity [147]. It remains elusive how trans-signaling leads to granulocyte recruitment since in models for acute inflammation, apoptosis of neutrophils leads to shedding of the IL-6R and subsequent recruitment of monocytes via trans-signaling [39]. The recruitment of granulocytes to the site of inflammation is not affected by blockage of IL-6 trans-signaling, therefore trans-signaling is unlikely to influence granulocytes infiltration [39]. Nevertheless, the current data indicate that IL-6 and the sIL-6R are involved in IBD, even though the precise role and mechanism is not elucidated, yet.

3.5.1 IL-6, but not the IL-6R is an important factor in IBD

In the last chapter of this doctoral thesis, the role of IL-6 and the IL-6R in a murine DSS-induced colitis model was investigated. Given that IL-6 trans-signaling plays a detrimental role in autoimmune diseases, we expected that IL-6 and the IL-6R are needed for full disease severity. Unlike this initial hypothesis, we demonstrated that the IL-6R does not play a role in DSS-induced colitis in mice.

In the course of UC, there is a complex interplay between tissue damage and regeneration. IL-6 plays an important role in wound healing [193], whereas the IL-6R only plays a minor role [121]. Therefore, we investigated the role of IL-6 in DSS-induced colitis in mice. Previous studies reported a role for IL-6, but depending on the settings, IL-6 had a detrimental or beneficial role [137,138]. High doses of DSS resulted in an increased mortality in female *Il-6*$^{+/+}$ compared to *Il-6*$^{-/-}$ mice [138]. Low doses of DSS resulted in an increased regeneration of male *Il-6*$^{+/+}$ compared to *Il-6*$^{-/-}$ mice, but with higher tumor formation as a long term

consequence [137]. In our study we mimicked the latter experiment. Therefore we used male *Il-6*$^{+/+}$ mice and a low dose of DSS. We neutralized IL-6 by intraperitoneal injections of neutralizing IL-6 antibody and compared the susceptibility to control animals. In contrast to the latter experiment, we found that inhibition of IL-6 signaling is beneficial for mice in a DSS-induced colitis model. This indicated that IL-6 signaling has detrimental effects in the course of disease. This is in part consistent with findings from other experiments where blockade of IL-6 trans-signaling by sgp130Fc decreased disease activity [147]. Blockage of IL-6 might therefore be a promising target in IBD.

3.5.2 IL-6 signaling devoid of the IL-6R? – IL-6/IL-6R double KO mice as a chance to get some answers

It was found that IL-6 has a detrimental role in a murine DSS-induced colitis model. It was also established that the IL-6R plays no such role. IL-6 binds to the membrane bound or soluble IL-6R, forming an IL-6/IL-6R or IL-6/sIL-6R complex [5], respectively. This complex then activates signal transduction via a gp130 homodimer (Figure 9A, B). Besides its function in IL-6 signaling, the IL-6R is also involved in signal transduction of IL-30 and CNTF [33,195]. IL-30 binds to the membrane bound or soluble IL-6R [33]. The complex subsequently activates a gp130 homodimer (Figure 9D, E) [33]. At high concentrations, IL-30 activates a gp130 homodimer without the IL-6R (Figure 9F) [33]. Another ligand for the IL-6R is CNTF [195]. This cytokine binds with a high affinity to the CNTF receptor (Figure 9I) [196]. CNTF can also bind to the membrane bound and the soluble IL-6R with a lower affinity than to the CNTF receptor (Figure 9G, H) [195]. After complex formation, both complexes recruit a heterodimer of gp130 and LIF receptor for signal transduction [195].

CNTF and IL-30 could therefore be the reason for the difference in susceptibility in DSS-induced colitis between mice lacking the IL-6R and mice lacking IL-6 signaling. In chapter 5 of this thesis, IL-6 was shown to worsen the course of disease in a DSS-induced colitis model. Surprisingly, lack of IL-6R did not affect susceptibility. Therefore, beneficial effects of the lack of IL-6 signaling could be compensated by negative effects of CNTF or IL-30 signaling in a complex with the IL-6R. A double deficient mouse, lacking IL-6 and the IL-6R would be a good tool to discriminate between the effects of IL-6 and IL-6R in a DSS-induced colitis model. Wt mice and IL-6R deficient mice show a normal susceptibility in this model of disease; lack of IL-6 leads to a decreased susceptibility. If there were any beneficial effects of either CNTF or IL-30 mediated exclusively in a complex with the IL-6R, these effects would be lost in IL-6R deficient mice. Also IL-6 mediated signals would be lost. Therefore, the

susceptibility should resemble to that of wt animals. In a double deficient mouse, IL-6, CNTF and IL-30 signals via the IL-6R are lost as well. Thus, a double deficient mouse should present the same susceptibility in a DSS-induced colitis model as the IL-6R deficient and the wt mouse do. It would also be possible that there is a yet unknown ligand for the IL-6R and that this ligand is compensating the negative effects of IL-6 in this model of disease (Table 2, Option 1).

In principle there could be another result when challenging the double deficient mouse: IL-30 and CNTF might have no influence on the susceptibility of mice in a DSS-induced colitis model in respect to the IL-6R. It is possible that IL-6 has another yet unknown receptor besides the IL-6R mediating the detrimental effects of IL-6 in a DSS-induced colitis model and the signaling mediated by IL-6 and the membrane bound or soluble IL-6R might not be important for the susceptibility. In this case, lack of IL-6 signaling would have positive effects in a DSS-induced colitis model. Lack of the IL-6R would lead to the same susceptibility wt animals show. IL-6 would no longer be able to induce signaling after forming the IL-6/(s)IL-6R complex which would not affect colitis, but signaling of IL-6 via its unknown receptor would not be abrogated and shows the same negative effects as it does in wt mice. So far, both models are consistent with the results for wt, IL-6 deficient and IL-6R deficient mice. The *Il-6*$^{-/-}$/*Il-6r*$^{-/-}$ mouse would render the models distinguishable. If IL-6 and the IL-6R lack both, the double deficient mouse would show the same susceptibility as the IL-6 deficient mouse does. Lack of IL-6 signaling via the complex with the IL-6R would have no effect, but lack of the detrimental signaling of IL-6 via the unknown receptor would decrease the susceptibility of mice compared to wt and IL-6R deficient mice (Table 2, Option 2).

These models highlight the importance of experiments with *Il-6*$^{-/-}$/*Il-6r*$^{-/-}$ mice. In wound healing, IL-6 was important for wound closure, but the IL-6R did not play a role [121]. The *Il-6*$^{-/-}$/*Il-6r*$^{-/-}$ mice showed the same phenotype as wt and *Il-6r*$^{-/-}$ mice, whereas *Il-6*$^{-/-}$ showed a delay in wound closure. In this model of tissue regeneration, the first of the two working models is preferable. Therefore, it was excluded by neutralizing antibodies that IL-30 is important for wound closure [121]. However, CNTF is unlikely to be the cytokine determining the difference between *Il-6*$^{-/-}$/*Il-6r*$^{-/-}$ and *Il-6*$^{-/-}$ mice, due to its low affinity to the (s)IL-6R [195].

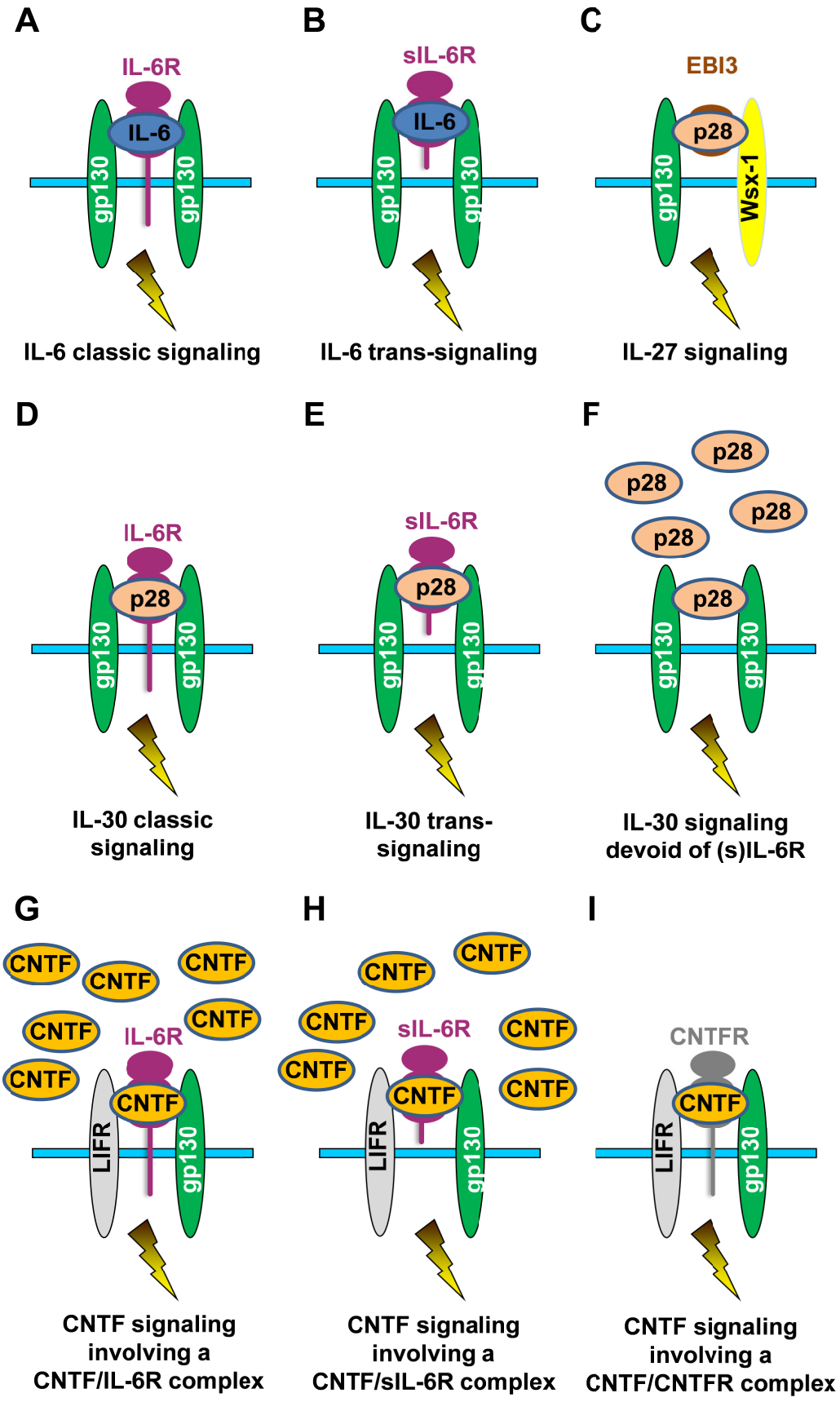

Figure 9: Overview over signals that could be involved in the IL-6 and IL-6R mediated susceptibility in colitis.

A) The IL-6/IL-6R complex binds to a gp130 homodimer inducing IL-6 classic signaling [28]. **B)** The IL-6/sIL-6R complex binds to a gp130 homodimer inducing IL-6 trans-signaling [28]. **C)** The complex of EBI3/p28 (IL-

27) binds to a heterodimer of Wsx-1/gp130 inducing IL-27 signaling [7]. **D)** IL-30 (p28) binds to the membrane bound IL-6R recruiting a homodimer of gp130 for classic signaling [8]. **E)** IL-30 binds to the soluble IL-6R recruiting a homodimer of gp130 for trans-signaling [33]. **F)** At high concentrations IL-30 can directly bind to a gp130 homodimer inducing signal transduction [33]. **G)** At high concentrations CNTF can bind to the membrane bound IL-6R recruiting a heterodimer of LIF receptor (LIFR) and gp130 for signal transduction [195]. **H)** At high concentrations CNTF can bind to the soluble IL-6R recruiting a heterodimer of LIFR/gp130 for signal transduction [195]. **I)** At low concentrations CNTF only binds to the CNTF receptor (CNTFR). The CNTF/CNTFR complex subsequently recruits a LIFR/gp130 heterodimer for signal transduction [196].

	Option 1			**Option 2**		
	IL-6 + IL-6R	**Unknown ligand + IL-6R**	**Susceptibility**	**IL-6 + IL-6R**	**IL-6 + Unknown receptor**	**Susceptibility**
Wild type	**-**	**+**	**normal**	**0**	**-**	**normal**
***Il-6*$^{-/-}$**	no signal	**+**	**reduced**	no signal	no signal	**reduced**
***Il-6r*$^{-/-}$**	no signal	no signal	**normal**	no signal	**-**	**normal**
***Il-6*$^{-/-}$ *Il-6r*$^{-/-}$**	no signal	no signal	**normal**	no signal	no signal	**reduced**

Table 2: Overview over the results and potential results of DSS-induced colitis studies in mice.
In principle there are two distinct possibilities how IL-6 and the IL-6R influence the susceptibility of mice challenged in a DSS-induced colitis model. The first possibility is, that IL-6 mediates a detrimental signal via the IL-6R which is compensated by a protective signal of an unknown ligand via the IL-6R. This results in a normal susceptibility. An IL-6 deficient mouse lacks the detrimental signal of IL-6 but keeps the protective signal of the unknown IL-6R ligand. The mouse has a reduced susceptibility. An IL-6R deficient mouse lacks both signals and therefore shows a normal susceptibility. If option 1 is correct, the double deficient mouse would have a normal susceptibility due to lack of beneficial and detrimental signals. The second possibility is, that IL-6 mediates an irrelevant signal via the IL-6R and a detrimental signal via an unknown receptor. This results in a normal susceptibility. An IL-6 deficient mouse lacks the irrelevant and the detrimental signal of IL-6, therefore its susceptibility is reduced. An IL-6R deficient mouse lacks the irrelevant signal of IL-6 via the IL-6R but not the detrimental signal of IL-6 via its unknown receptor resulting in a normal susceptibility. If option 2 is correct, the double deficient mouse would have a reduced susceptibility due to lack of irrelevant and detrimental signals.

It is important to mention that the results for wound healing can only be considered as evidence for the potential situation in DSS-induced colitis. The two animal models are comparable in some aspects, but not the same. Principle mechanisms could be similar. In both models, tissue damage is set under non-sterile conditions. On the skin of mice, there are commensal bacteria as in the colon. Therefore, the immune system is involved in both models for limiting bacterial burden. At the same time, regeneration processes take place.

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5 ABBREVIATIONS

%	percent
3'UTR	3‘ untranslated region
5'UTR	5‘ untranslated region
aa	amino acid
ADAM10	A Disintegrin And Metalloprotease 10
ADAM17	A Disintegrin And Metalloprotease 17
AOM	azoxymethane
APC	adenoma polyposis coli protein
APRE	acute phase responsive element
ATG16L1	autophagy-related16-like 1
Ba/F3	Murine pro B-cells cell line
bp	base pair
BSF-2	B cell differentiation factor 2
CBM	cytokine binding module
CD	Crohn's disease
CK2	protein kinase II
CLC	cardiotrophin like cytokine
CNTF	ciliary neurotrophic factor
CRC	colorectal cancer
CRP	C-reactive protein
CT-1	cardiotrophin-1
D	domain
DSS	dextran sodium sulfate
ΔYY	deletion of Tyr^{186} to Tyr^{190}
E	exon

EBI3	Epstein-Barr virus induced protein 3
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
et. al.	and others
EU	European Union
F	phenylalanine
Fc	constant fragment of an antibody
FN	fibronectin
gp130	glycoprotein 130
gp130ΔSTAT	gp130 variant with deletion of all STAT binding sites
gp130F759	gp130 variant with exchange of amino acid 759 to phenylalanine
GPL	gp130-like-receptor
h	hour
hn	heteronuclear
Hyper-IL-6	fusion protein of IL-6 and the sIL-6R
IBD	inflammatory bowel disease
ICD	intracellular domain
Ig	immunoglobulin
IHCA	inflammatory hepatocellular adenoma
IL	Interleukin
IL-11R	Interleukin-11 receptor
IL-23R	Interleukin-23 receptor
IL-6$^{-/-}$	Interleukin-6 deficient
IL-6$^{-/-}$/IL-6R$^{-/-}$	Interleukin-6 and Interleukin-6 receptor deficient
IL-6R	Interleukin-6 receptor
IL-6R$^{-/-}$	Interleukin-6 receptor deficient
iNKT cell	invariant natural killer T cell

IPA	intronic polyadenylation
JAK	Janus kinase
JAM-A	junction adhesion molecule A
kDa	kilo dalton
KO	knock out
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
m	messenger
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemo-attractant protein-1
MDR	multiple drug resistant
MUC2	mucin 2
myc-His	v-myc avian myelocytomatosis viral oncogene homolog-histidine
NFκB	nuclear factor kappa-light-chain-enhancer of activated B-cells
ng	nanogram
NOD2/CARD15	nucleotide-binding oligomerization domain 2/caspase recruitment domain 15
NPN	neuropoietin
OSM	oncostatin M
OSMR	oncostatin M receptor
p28	protein 28, also termed IL-30 as a monomer
PAS	polyadenylation site
PBMC	peripheral blood mononuclear cells
Phe	phenylalanine
PI3K	phosphatidyl inositol 3 kinase
PRR	pattern recognition receptor
Q	glutamine
RA	rheumatoid arthritis

RAG-2	recombination-activating gene 2
RAPS	rheumatoid arthritis antigenic peptide-bearing soluble form
RNA	ribonucleic acid
RNP	ribonucleic particle
RT-PCR	reverse transcription polymerase chain reaction
S	signal peptide
SAM	Senescence accelerated mouse
SAMP1/Yit	a special mouse strain
SCID	severe combined immunodeficient
sgp130	soluble gp130
sgp130-Diamant	soluble gp130 isoform identified by Diamant *et. al.*
sgp130-E10	soluble gp130-Exon 10
sgp130-Sharkey	soluble gp130 isoform identified by Sharkey *et. al.*
SHP-2	Src homology 2 domain-containing protein tyrosine phosphatase-2
sIL-6R	soluble Interleukin-6 receptor
sn	small nuclear
SNP	single nucleotide polymorphism
SOCS	suppressors of cytokine signaling
STAT	signal transducer and activator of transcription
TCCR	T cell cytokine receptor
T_H17	T helper 17
TM	transmembrane domain
TNBS	trinitro benzene sulfonic acid
T_{reg}	regulatory T cell
Tyr	tyrosine
UC	ulcerative colitis
US	United States

WSX-1	receptor for IL-27 as part of the Wsx-1/gp130 heterodimer
wt	wild type
X	any amino acid
XBP1	X-box binding protein 1
Y	tyrosine
Yit	Yakult Central Institute for Microbiological Research Tokyo

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7 STATEMENT

Hiermit erkläre ich an Eides Statt, dass ich die hier vorgelegte Dissertation selbstständig und ohne unerlaubte Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt habe. Die Dissertation wurde in der vorgelegten oder in einer ähnlichen Form noch bei keiner anderen Institution eingereicht. Es wurden bisher keine erfolglosen Promotionsversuche von mir unternommen.

Düsseldorf, im März 2014

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Jan Sommer