Immune-based therapies in ovarian cancer

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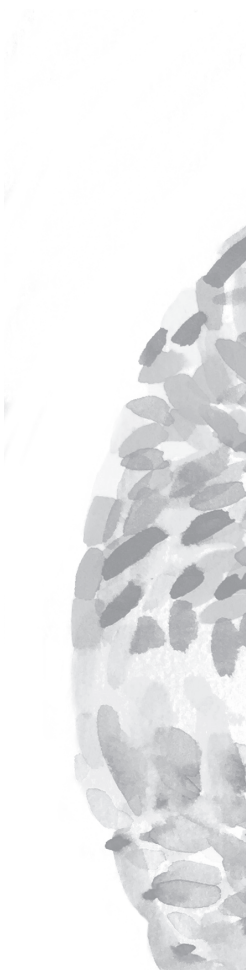

CHAPTER 2

Interleukin-6/interleukin-6 receptor pathway as a new therapy target in ovarian cancer

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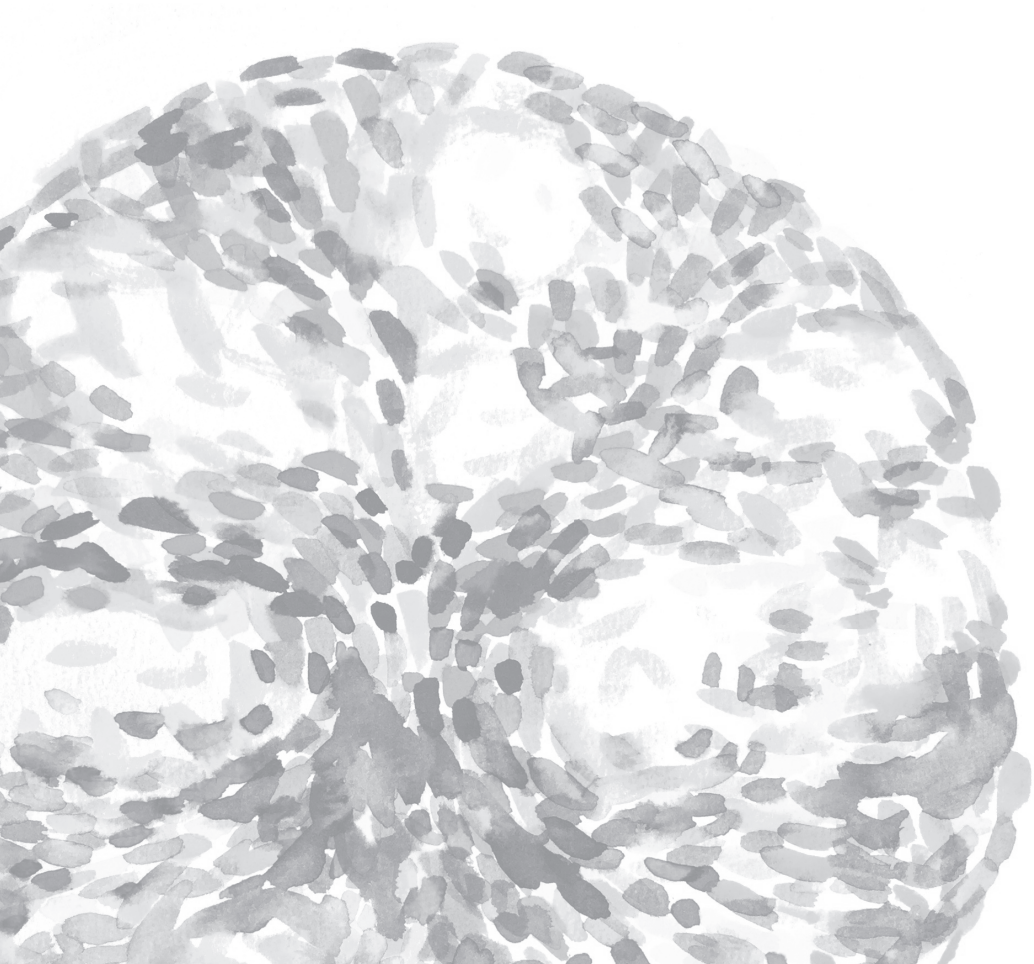

ABSTRACT

Epithelial ovarian cancer is a major problem as about 75% of patients develop recurrence after initial primary treatment and tumors are often chemoresistant. This article reviews the role of the interleukin-6 (IL-6) in chemoresistance and suppression of tumor immunity in ovarian cancer and provides the rationale for modulating the IL-6/ IL-6 receptor (IL-6R) induced pathway as a potential new target for the treatment of ovarian cancer. IL-6 is elevated in serum and ascites of ovarian cancer patients and increased IL-6 levels correlate with chemoresistance and poor prognosis in these patients. IL-6 induced Jak/Stat3, Ras/MEK/ERK and PI3K/Ras signaling pathways lead to cell survival, proliferation, angiogenesis, and confers resistance to apoptosis induced by conventional therapies. Furthermore, IL-6 induces tumor-promoting macrophages which are known to foster tumor growth and suppress local immunity. However, direct proof of the clinical impact of IL-6 blocking on disease progression is missing necessiting further studies in which the IL-6(R) pathway is modulated and its clinical impact on (epithelial) ovarian cancer is tested.

INTRODUCTION

Ovarian cancer accounts for around 200,000 new cases each year worldwide [1]. Conventional treatment results are poor. Notwithstanding the good initial response to primary therapy, aggressive debulking surgery combined with chemotherapy (carboplatin and paclitaxel), 75% of the patients with advanced disease will develop recurrent disease [1], causing approximately 60-80% of patients to die within 5 years of initial diagnosis [1]. Hence, there is a pressing need to identify novel targeted therapies or therapy combinations that (re)sentisize tumor cells to existing chemotherapy.

Interleukin-6 (IL-6) seems a promising new target for therapy in ovarian cancer; up-regulation of IL-6 in serum and ascites of patients is associated with disease progression and resistance to chemotherapy in patients with ovarian cancer [2-5].

Additionally, ovarian cancer is highly immunogenic [6-11]; striking correlations exist between host immunity, tumor infiltration with effector T cells and a favorable clinical outcome [11]. IL-6 is one of the major immuneregulatory cytokines present in the ovarian microenvironment and has several properties by which it can influence the tumor environment towards an immunosuppressive environment [12].

Collectively, the modulation of IL-6 induced signaling pathway may provide a novel and effective approach to treat human ovarian carcinoma. In this review, we focus on the role of IL-6 in epithelial ovarian cancer and detail its expression and regulation, its function in the tumor immunity, and review the current and future possibilities of IL-6/IL-6R inhibition in ovarian cancer.

SEARCH STRATEGY

A systematic literature search within the PubMed-database was conducted in July 2011. The following keywords were used, alone or in combination: (*ovarian) cancer, interleukin-6, immunotherapy, IL-6(R), sIL-6R, Jak/Stat3, Ras/MEK/ERK, PI3K/Akt, CNTO328, siltuximab, monoclonal antibody and tocilizumab*. Results were assessed by reviewing titles and abstracts, and relevant articles were retrieved. Cited references in these articles were used to find further relevant articles. Only articles in English were selected. Eligible abstracts had to include epithelial ovarian cancer; this is the most common type of ovarian cancer [1] and other types of ovarian cancer behave differently and were therefore excluded [13]. Additionally, a search within the American Society of Clinical Oncology (ASCO) abstracts database was conducted using the previously mentioned terms. Abstracts from ASCO Annual Meetings of the years 2008 to 2010 that met the above criteria were included in this review.

OVARIAN CANCER

Ovarian cancer has a dismal prognosis and there is a pressing need to identify novel targets and therapies. Since most patients are asymptomatic until the disease has metastasized, two-thirds are diagnosed with advanced stage. For the same reason, ovarian cancer has the highest fatality-to-case ratio of all gynecologic malignancies. Worldwide, of the approximately 200,000 women diagnosed with ovarian cancer, about 115,000 die from this disease yearly [1]. There has been a decline of less than 1 percent in the incidence of ovarian cancer in the last two decades, and the mortality rate is largely unchanged [14]. Conventional therapy for ovarian cancer is surgical tumor cytoreduction followed by a combination of platinum and nonplatinum (taxane-based) chemotherapy, such as carboplatin and paclitaxel. Initially, this results in a clinical remission in up to 75% of cases. However, 75% of the responders will relapse within 18 to 28 months and only 20% to 40% of women will survive beyond five years [1]. Ovarian cancer tends to develop from three kinds of tissue: approximately 85 to 95% from epithelial cells, 5 to 8% from stromal cells, and 3 to 5% from germ cells. The type of ovarian tumor varies depending on the patient's age. Epithelial cell tumors usually occur in women older than 50 years. Ovarian cancer primarily spreads locally to the opposite ovary and the uterus, and subsequently intraperitoneally. Distant metastases are rare, but may occur [15].

The clinical outcome of ovarian cancer depends on the immune response. Ovarian cancer is generally known as highly immunogenic and striking correlations exist between the infiltration of tumors by immune cells and clinical outcome. Strong infiltration with CD8+ T cells is associated with a more favorable overall survival after treatment whereas infiltration by immune suppressive regulatory T cells correlates with a worse prognosis [6-11]. The balance between immune-activating and immune-suppressing mechanisms determines the final clinical outcome.

Immune cells, including B and T lymphocytes, macrophages and dendritic cells (DC), may specifically infiltrate tumors tissue as compared to normal healthy tissue [16]. T-lymphocytes are a major component of cellular immune response, required for anti-cancer immunity. There are two major lines of T cells: cluster of differentiation 4 positive (CD4+) T cells and CD8+ T cells. CD4+ T cells come in many flavors, including so-called helper and regulatory T cells. In cancer, IFNg-producing T-helper type 1 cells are important to activate professional antigen-presenting cells (APCs), such as DC that present the cancer–antigen as this allows the DC to fully activate CD8+ T cells. Tumor-specific CD4+ Th1 cells are needed to sustain the CD8+ T cell response by the production of IL-2, but also to allow the CD8+ T cells to infiltrate tumors and to exert their effector function against the cancer cells. T-helper type 2 cells are known to sustain the effector function of B cells, and their interaction with APC may – depending on the situation – result in a tumor-rejecting or tumor-promoting activities. The CD4+ T helper cells and the CD8+ cytotoxic T-cells are both kept under control by

CD4+ regulatory T cells (Tregs). More recently, CD8+ Tregs have also been identified. Tumor infiltrating APCs comprise macrophages, which are usually the most abundant immune population present in the tumor microenvironment. Macrophages infiltrating cancer tissue are referred to as tumor-associated macrophages (TAM) [17]. They can differentiate from monocytes into tumor-rejecting M1 or tumor-promoting M2 macrophages. Depending on the environmental cues they encounter M1 macrophages, producing IL-12 needed to stimulate anti-tumor immunity. They have the potential to kill tumor cells, and can ingest (necrotic) tumor cells in order to present tumor (associated) antigens to the infiltrated T cells resulting in inhibition of tumor growth or regression. Instead, the M2 macrophages, which produce amongst others IL-10, suppress adaptive immunity and promote matrix remodeling, tumor growth and survival, invasion and metastasis as well as angiogenesis [18]. In addition, the tumor microenvironment contains a large number of immunosuppressive cytokines (eg TGF-β and IL-10) and soluble mediators (eg prostanoids such as Prostaglandin E2 [PgE2] and IL-6) produced by tumor cells and/or immune cells resulting in suppression of the anti-tumor response.

Current new strategies are based upon this knowledge: vaccines are developed against known tumor-associated antigens, e.g. HER2/neu, NY-ESO-1 and p53 which are expressed by ovarian cancer cells and can serve as targets for humoral and cellular immune responses. Several phase I/II studies were performed using HER2/neu derived peptide vaccination in patients with HER2/neu overexpressing tumors. The magnitude of the T cell response appears to correlate favorably with the clinical response [19;20]. Antibody based vaccines, for instance antibodies recognizing the surface glycoprotein CA-125, are a main focus as well. In a phase II study oregovomab (a monoclonal antibody that binds to CA-125 with high affinity) was combined with chemotherapy. Of all patients, 63% showed T cell immunity against the autologous tumor and this significantly improved survival of the patients. However, 50% of the patients showed an humoral response directed against oregovomab [21-25]. Properly activated DCs have the potential to induce antigen specific anti-tumor immunity, therefore infusion of autologous DCs pulsed with tumor associated antigens (eg peptides) have been investigated in several pilot studies. This approach demonstrated to be capable to induce antigen-specific cytotoxic T cell responses [26-28]. Similarly a p53 synthetic long peptide vaccine was shown to be highly immunogenic as well [29].

The fact that both the presence of TILs correlated strongly with survival and ovarian cancer cells express MHC class I-peptide complexes, which can be recognized by CD8+ T lymphocytes, makes adoptive T cell therapy an interesting approach. Unfortunatley, adoptive CD8+ T cell transfers appeared to reduce the clinical efficacy in ovarian cancer versus similar cell transfers in other epithelial carcinomas [30].

It is highly likely that the dynamic interaction between host immunity and cancer is modulated at multiple levels, especially within the tumormicroenvironment where these T cells have to exert their function. In order to improve the outcome of T cell based therapies

one might consider to deplete the various sorts of immune suppressing cells and/or to focus on blocking the effects of soluble mediators. We focus on the role of IL-6, which is known to polarize macrophages towards the often in tumors found tumor-promoting type of macrophage, and mediates also other mechanisms leading towards an immunosuppressive tumor microenvironment. [*see IL-6 involvement in anti-cancer immunity*].

INTERLEUKIN-6

IL-6, a 185 amino acid polypeptide, is a pleiotropic cytokine that plays a major role in the response to infection and is involved in the immune response, inflammation, and hematopoiesis. IL-6 is frequently up-regulated in acute and chronic inflammatory conditions, including many cancers and especially ovarian cancer. In homeostatic conditions, IL-6 levels are low, whereas, under stress conditions, levels of IL-6 rise quickly in the serum. IL-6 can be produced by various immune and non-immune cells such as T cells, B cells, macrophages, monocytes, fibroblasts, endothelial cells and tumor cells [31]. IL-6 belongs to the IL-6 family of cytokines which share remarkably similar structural features both for cytokines and the class I cytokine receptors to which they bind. These similarities also extend to the sequential clustering events leading to signal transduction. The closest members of the family include leukemia inhibitory factor (LIF), cillary neutrophic factor (CNTF), oncostatin M and IL-11 [32]. Increased production of IL-6 has been implicated in various disease processes, including Alzheimer's disease, autoimmunity (e.g. rheumatoid arthritis), inflammation, myocardial infarction, Paget's disease, osteoporosis, B-cell malignancies and solid tumors (e.g. renal cell cancer and ovarian cancer) [33].

IL-6 signaling. The IL-6 receptor system consists of two polypeptide chains: a specific ligand binding 80 kDa IL-6 receptor (α-chain; glycoprotein 80 or gp80; CD126) and a signal transducing component consisting of two molecules of a 130 kDa, gp130 (β-chain, CD130) [31]. IL-6 can act on other cells locally or systemically by two different mechanisms. In the first, called classical pathway, IL-6 signaling occurs through the transmembrane IL-6 receptor (IL-6R), consisting of the specific α-chain and the shared β-signaling receptor gp130 [**Figure 1**] [34].

Dimerization of gp130 leads to activation of several pathways which will be further described below. Secondly, cells that do not express IL-6R can respond to IL-6 when it is already associated with a soluble IL-6 receptor (sIL-6R) in a process called trans-signaling (or alternative pathway) [**Figure 2**] [35;36].

These sIL-6R isoforms are generated by two independent mechanisms: limited proteolysis of the membrane protein (10%) and translation from an alternatively spliced mRNA 90% [37], yielding a protein that differs at its COOH terminus by 14 amino acids residues [38]. The differentially spliced isoform lacks a transmembrane domain and is secreted as a soluble,

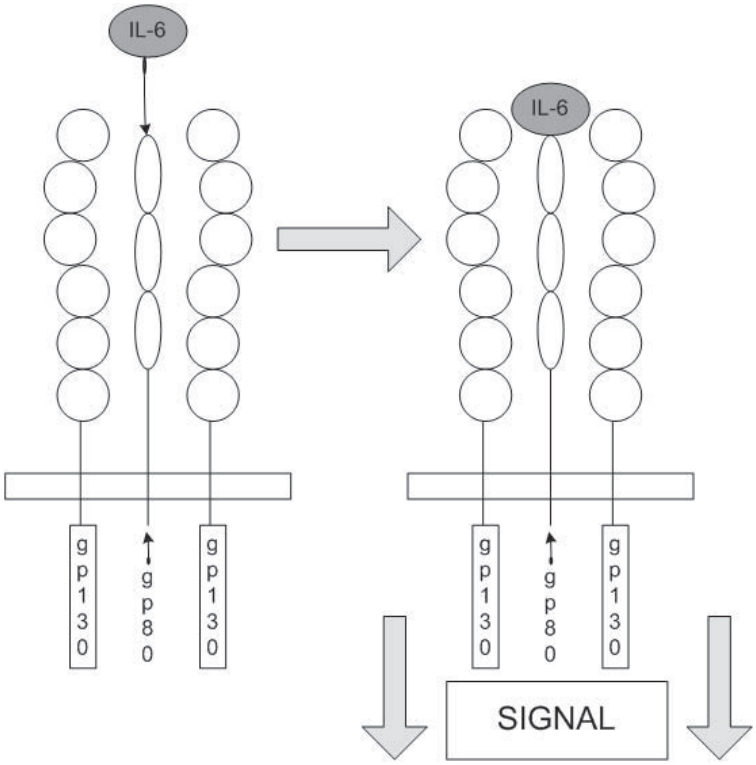

Figure 1. Classical pathway.
IL-6 binds to IL-6R and subsequently the two gp130 molecules are recruited. Ligand binding to gp130 leads to activation of three different pathways leading to cell survival, cell proliferation, angiogenesis and anti-apoptosis Fig. (**3**).

functional receptor [39]. The proteolytic enzymes responsible for cleavage of full-length IL-6R are desintegrin and metalloprotease domain (ADAM)-17, also known as tumor necrosis factor alpha-converting enzyme and, to a lesser extent, ADAM10. These sheddases cleave full-length IL-6R in a membrane proximal site to release a soluble functional receptor [34]. The sIL-6R can bind to its ligand IL-6 with comparable affinity as the membrane-bound IL-6R [40]. This alternative pathway seems responsible for the majority of the effects of IL-6 [41],[42].

Signaling occurs when either IL-6 binds to the membrane-bound IL-6R or when the soluble IL-6R α-chain subsequently recruit two gp130 molecules (β-chain) and binds IL-6 into the groove formed by the α-chain and the two gp130 molecules. This leads to intracellular signaling and the activation of three different pathways controlling cell survival, proliferation and angiogenesis [**Figure 3**] [43]. IL-6 can increase this activity both in tumor cells and in tumor-associated immune cells [44].

The first pathway induced by IL-6 is the activation of Janus tyrosine kinases (JAK1, JAK2 and TYK2) and subsequent the signal transducer activator of transcription (Stat). Stat3 is the

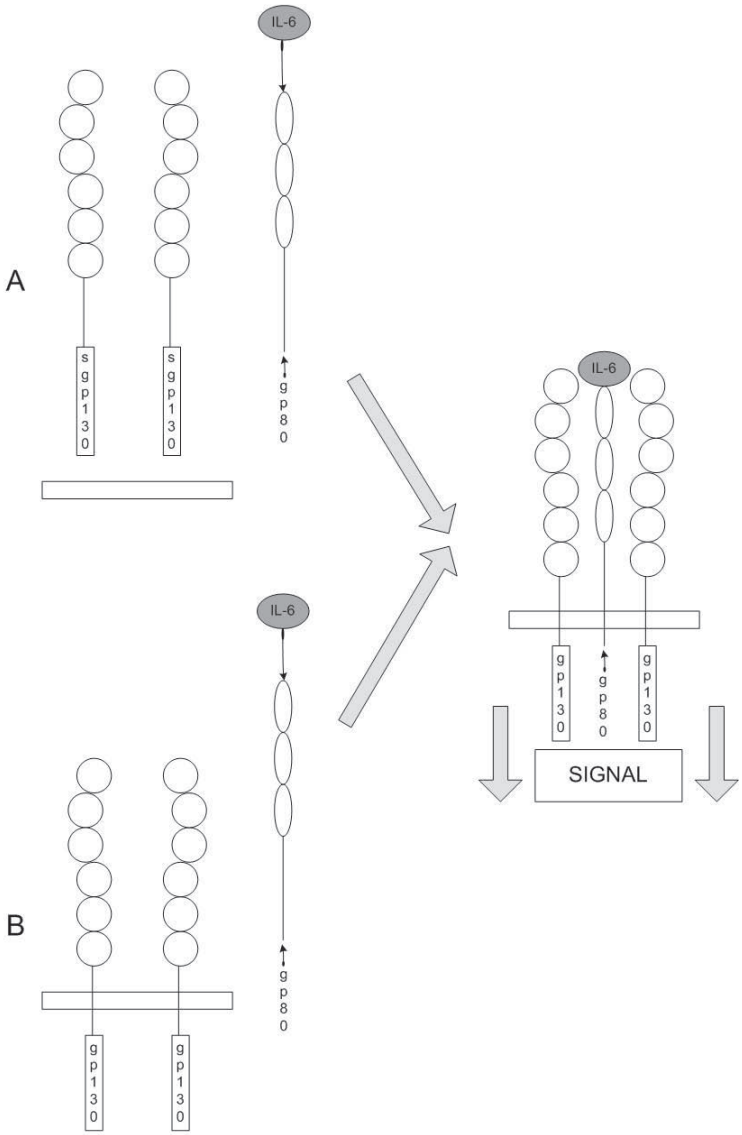

Figure 2. Alternative pathway (trans-signaling).
Cells that are deficient in or lack IL-6R can respond to IL-6 with a soluble IL-6 receptor (sIL-6R). Only the α-chain can be soluble (**A**) or both the α- and β-chain of the IL-6R (**B**). When the sIL-6R and IL-6 complex recruit two gp130 molecules, the activation cascade described in figure 3 is induced.

major Stat induced by IL-6, and its nuclear translocation induces a transcriptional program resulting in inflammation, cell survival, or differentiation, depending on the cellular context [45;46]. Binding of Stat3 to a specific DNA domain promotes the expression of survival proteins [i.e. survivin, X-linked inhibitor of apoptosis (XIAP), B-cell lymphoma-2 (Bcl-2), B-cell

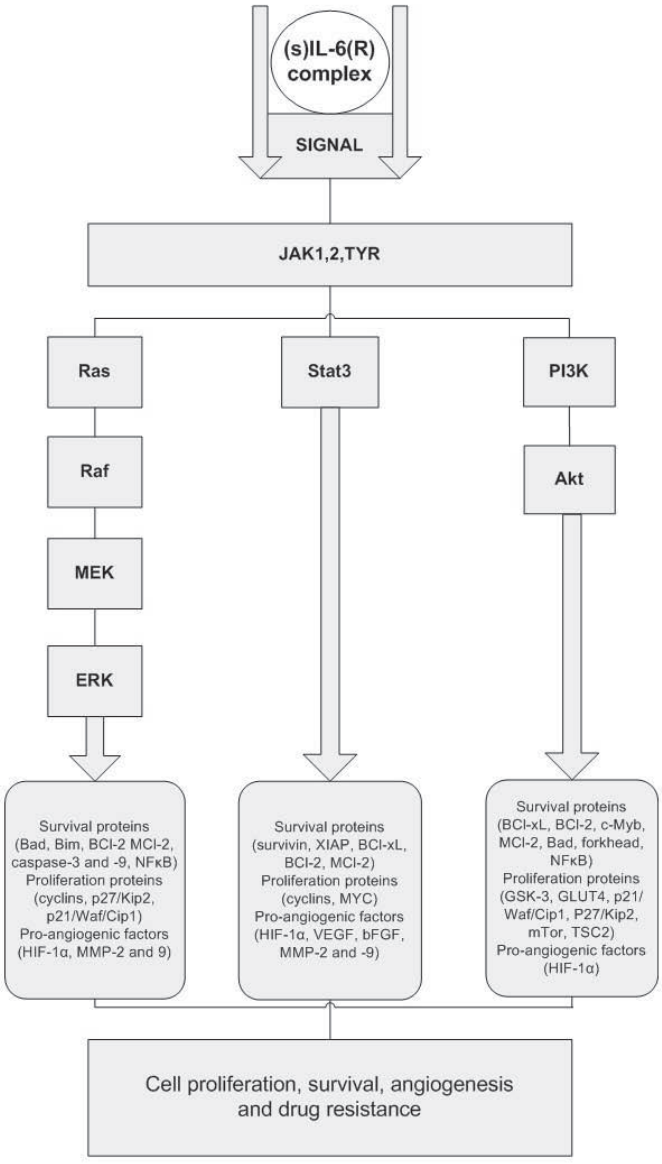

Figure 3. Signaling pathways induced by IL-6.
Ligand binding of IL-6 leads to activation of Janus tyrosine kinases and subsequent activation of the three different pathways controlling cell survival, proliferation, angiogenesis and anti-apoptosis.

lymphoma-xL (Bcl-xL), and myeloid leukemia cell protein-1 (Mcl-1)], proteins involved in cell proliferation [i.e. cyclins and avian myelocytosis virus oncogene cellular homolog (MYC)] and proangiogenic factors [i.e. hypoxia-inducible factor (HIF)-1α, vascular endothelial

growth factor (VEGF), basic fibroblast growth factor (bFGF), and matrix metalloproteinase (MMP)-2 and -9].

The rat sarcoma (Ras) oncogene is also activated through the activation of Janus tyrosine kinases by IL-6 and promotes its translocation to the plasma membrane where it activates rapidly accelerated fibrosarcoma (Raf) kinase. Raf in turn activates mitogen-activated protein kinases (MAPK) containing MAPK kinase (MEK) and extra-cellular signal related kinase (ERK1/2). The protein kinases then phosphorylate the nuclear factor for IL-6 (NF-IL-6), a process that is essential for DNA binding. NF-IL-6 binds and activates the promoter regions of various acute phase proteins [47]. The Raf/MEK/ERK pathway also has profound effects on the regulation of apoptosis by the post-translational phosphorylation of apoptotic regulatory molecules including Bad, Bim (members of Bcl-2 family), Mcl-1, caspase 9 and Bcl-2. This pathway has diverse effects which can regulate cell cycle progression, apoptosis or differentiation[48-50].

A third pathway activated by IL-6 (again by the activation of Janus tyrosine kinases) is the phosphoinositol 3 kinase (PI3K)-protein kinase B/cellular homolog of the oncogene of retrovirus Akt B (PkB/Akt) pathway. Activation of Akt plays a pivotal role in fundamental cellular functions such as cell proliferation and survival by phosphorylating a variety of substrates [51]; activated Akt protein modulates the function of the regulation of cell proliferation (i.e. glycogen synthase kinase-3 [GSK-3], membrane translocation of the glucose transporter GLUT4, Cyclin-dependent kinase inhibitors [P21/Waf1/Cip1 and P27/Kip2], mammalian target of rapamycin (mTOR) and tuberous sclerosis complex 2 [TSC2]). Cell survival is regulated by Akt by phosphorylating and inactivating pro-apoptotic proteins such as Bad, which controls the release of cytochrome C from mitchondria. Phosphorylation of Akt leads to the nuclear translocation and activation of nuclear factor-kappaB (NFκB), and transcription of NFκB-dependent prosurvival genes [i.e. Bcl-xL, caspase inhibitors and myeloblastosis oncogene (c-Myb)]. Phosphorylation of the forkhead family of transcription factors inhibits transcription of pro-apoptotic genes [i.e. Fas Ligand (FasL), insulin-like growth factor-binding protein-1(IGFBP-1) and Bim (B cell lymphoma 2-interacting (Bcl2-interacting) mediator)]. At last, Akt phosphorylation leads to nuclear translocation and activation of NFκB, and transcription of NFκB-dependent prosurvival genes [i.e. Bcl-xL, caspase inhibitors and c-Myb] and anti-apoptotic genes [i.e. Bcl-2 and Mcl-1] [51].

IL-6 regulation. The exact mechanism of regulation of IL-6 is yet unknown. A mechanism that might contribute to IL-6 over-expression includes methylation of suppressor of cytokine signaling (SOCS) proteins, inhibitors of cytokine signaling pathways and regulators of T-cell, macrophage and DC activation [52]. The classical pathway of IL-6 signaling systems is regulated by negative feedback by SOCS and the protein inhibitors of activated Stats (PIAS). IL-6/IL-6R interaction leads to activation of Stat3, which then targets SOCS-1. The SOCS-1 molecule then binds to the JAK tyrosine kinase and acts as a negative regulator of gp130 signal transduction [53]. SOCS3 has been shown to be a key regulator for the divergent

activity of IL-6. SOCS3 protein is strongly induced by both IL-6 and IL-10 in the presence of lipopolysaccharide (LPS), but IL-6 signaling is selectively inhibited owing to the binding of SOCS3 to the IL-6R subunit gp130. Therefore, Stat3 activation is transient in response to IL-6 [52].

Mutations in p53 and the retinoblastoma gene product have been implicated in the regulation of IL-6. The IL-6 promotor is inhibited by p53 and the retinoblastoma (Rb) gene product [54;55]. Wild-type p53 protein inhibited IL-6 promoter activity while mutant p53 appeared to induce IL-6 promotor activity [56]. The over-expression of IL-6 in ovarian cancer may occur as a result of the loss of one of these negative regulators of transcription.

Moreover, PgE2, which is believed to increase aromatase expression through several aromatase promotors, may act in part by stimulating IL-6 production in fibroblasts [57-59].

IL-6 INVOLVEMENT IN OVARIAN CANCER

Several preclinical studies have explored how IL-6 is regulated in ovarian cancer cell lines. It was initially thought that ovarian cancer cells produced their own IL-6, which perpetuated the malignancy through autocrine production [60;61]. However, there also seems to be a paracrine role for IL-6 as some ovarian cancer cells express an IL-6 receptor but do not secrete IL-6 [60]. Notably, IL-6 is constitutively produced by TAMs in ovarian carcinomas [62].

The relevance of IL-6 in ovarian carcinoma is suggested by several preclinical and clinical observations. First, we describe the clinical findings of IL-6 in ovarian cancer and then detail this involvement with preclinical evidence.

IL-6: a prognostic factor? **Table 1** gives an overview of the investigations that define the role of IL-6 in ovarian cancer. With the exception of the study of Plante *et al*. [63] the majority of studies suggest a correlation in serum and ascites IL-6 levels with poor prognosis. Plante *et al* [63] found no significant difference in survival time, although they did found a trend towards a higher level of IL-6 in patients who did not respond to chemotherapy.

Although serum IL-6 levels are correlated with a poor prognosis in ovarian cancer patients, it does not appear to be as sensitive or useful as a tumor marker as the cancer antigen 125 (CA125) [64;66] . No linear correlation between IL-6 and CA125 was observed [2;63;66]. IL-6 sensitivity was lower than that of CA125, and the combination of both assays did not increase the sensitivity of CA125 alone [66]. The combination of serum IL-6 with serum CA125 levels only slightly increased the overall sensitivity compared to CA125 alone, 92% vs 87% respectively. Postoperative IL-6 levels were shown to be less sensitive than CA125 levels in detecting residual disease after surgery [66].

Levels of sIL-6R, however, were significantly elevated in malignant ascites from patients with ovarian cancer and were associated with poor clinical outcome. A remarkable increase

Table 1. Overview of all studies investigating IL-6 levels in serum and ascites of patients with ovarian cancer

Year of publication	Author	n	Disease stage	Main Conclusions	p-value
1997	Tempfer *et al*.[64]	73	FIGO I-IV	Elevated IL-6 serum levels are significantly correlated with poorer PFS free* and OS**	* 0.003 ** 0.01
1995	Scambia *et al*.[4]	114	FIGO I-IV	Elevated IL-6 serum levels are significantly correlated with a poor prognosis	0.0009
1994	Schröder *et al*.[65]	67	FIGO I-IV	Patients with figo stage III/IV demonstrated higher levels IL-6 in serum (and ascites) than those with figo stage I/II	-
1994	Scambia *et al*.[66]	45	FIGO I-IV	Higher IL-6 levels were found in patients unresponsive to chemotherapy, as compared to the responders. Increased IL-6 serum levels correlate with negative prognosis	< 0.0004
1994	Plante *et al*.[63]	70	FIGO I-IV	No significant difference in OS, tumor stage, grade, histologic findings, residual tumor, serum CA125 levels. Responders to chemotherapy tender to have lower ascites levels of IL-6 compared to not-responders	NS
1993	Moradi *et al*.[67]	?	FIGO I-IV	IL-6 levels are elevated in serum and ascites levels	< 0.0001
1991	Berek *et al*.[2]	36	FIGO I-IV	Elevated IL-6 serum levels are significantly correlated with tumor burden, clinical disease status and OS	< 0.001

FIGO: international federation of gynecology and obstetrics
PFS: progression free survival
OS: overall survival

in sIL-6R was observed in malignant ovarian ascites as compared with those in peritoneal fluid from benign ovarian tumors (p=0.0046) [41].

IL-6 involvement in tumor progression. IL-6 has a growth stimulatory effect through the activation of the several signaling pathways leading to the transcription of cell survival and proliferation proteins [**Figure 3**].

In ovarian cancer cells, the IL-6 induced Stat3 signaling pathway is constitutively activated and is more active than in normal ovary cells. Stat3 signaling directly contributes to oncogenesis and maintenance of the malignant state [58;68]. Constitutive activation of Stat3 was accompanied by over-expression of Bcl-xL and cyclin D1 in several ovarian cancer cell lines and is associated with cancer progression [69].

Angiogenesis is a key rate-limiting step in the growth and dissemination of malignant tumors. In 2005 the role of IL-6 in the angiogenesis observed during the progression of ovarian carcinoma was first described. IL-6 seemed a potent inducer of the formation of new blood vessels; by stimulating cells with endogenous protein and measuring their migration in a

Boyden chamber assay, IL-6 was significantly found to enhance endothelial cell migration, a key step in the process of angiogenesis [70]. An increase in phosphorylated nuclear Stat3 levels subsequently upregulates proangiogenic molecules, including vascular endothelial cell growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9), thus contributing to angiogenesis and metastasis [45]. This process involves local tumor expansion through adjacent normal tissue, invasion of metastatic cells into vessels and of lymphatic tissue and extravasation at distant sites. IL-6 can enhance this expression through activation of the Stat3 or Ras/MEK/ERK-pathways, leading to upregulation of MMP-2 and -9 [44;59]. One of the proteins activated by all the IL-6 induced pathways is HIF-1α, which has been reported to allow the survival and proliferation of ovarian cancer cells through its angiogenic properties and transactivation during tumor progression [71].

IL-6 involvement in chemoresistance. One of the major obstacles in the treatment of ovarian cancer is the development of chemoresistance. Despite the good initial response to primary chemotherapy, responses are generally short lived and the ovarian cancer will recur with chemoresistant features. The molecular mechanism of chemoresistance in ovarian cancer is multifactorial and still not completely understood [72]. Various mechanisms have been proposed for chemoresistance in ovarian cancer. Mechanisms of chemoresistance that are associated with IL-6 induced pathways are discussed below.

Recently, Wang *et al* [73] studied the role of IL-6 expression in the acquisition of the chemoresistant phenotype and the underlying mechanisms of drug resistance. They reported that both exogenous (treatment with IL-6) and endogenous IL-6 (transfected cells with plasmid encoding for sense IL6) induced chemotherapy resistance in non-IL-6 overexpressing ovarian cancer cells. Deleting endogenous expression promoted the sensitivity. They suggested that the autocrine production of IL-6 by ovarian cancer cells promotes resistance of these cells to chemotherapy through decrease of proteolytic activation of caspase-3, thereby preventing apoptosis. They demonstrated that IL-6 induced resistance of ovarian cancer cells may be associated with Stat3 induced up-regulation of both multi-drug resistance-related genes [MDR1 and glutathione S transferase pi (GSTpi)] and apoptosis inhibitory proteins (Bcl-2, Bcl-xL and XIAP), as well as activation of Ras/MEK/ERK and PI3K/Akt signaling.

Generation of paclitaxel-resistant sublines of ovarian cancer cell lines has been associated with increased IL-6 mRNA expression and protein secretion [74]. Stat3 was often over-expressed and activated in many paclitaxel resistant ovarian cancer cells as compared with parental cell lines that were paclitaxel naïve. Stat3 inhibition increased paclitaxel induced apoptosis. Development of paclitaxel resistance in vitro is often accompanied with increased expression of Stat3 and downstream activation of Stat3 dependent genes. Stat3 decoy oligodeoxynucleotide inhibiting the function of Stat3, inhibited cancer cell invasive power [via downregulation of extracellular matrix metalloproteinase inducer (EMMPRIN)] and enhanced sensitivity to paclitaxel [via downregulation of MDR-1 and inhibition of phosphorylated Ak] [75][76]. Through induction of Stat3, XIAP - which is a direct inhibitor

of caspase-3,-7,-9, Fas, FasL and pro- and anti-apoptotic proteins – is expressed in ovarian cancer cells. This expression and downregulation of FasL are linked to chemoresistance in ovarian carcinoma cells [77-79]

HIF-1α, important in the survival and proliferation of ovarian cancer cells, was also found to have a pivotal role in chemoresistance [80]. Many of these HIF-1α -inducible genes directly or indirectly mediate chemoresistance, such as vascular endothelial growth factor (VEGF), Glut-1, MDR-1 and Bcl-2 [81;82]. Hypoxia significantly decreases the sensitivity of ovarian cancer cells to cisplatin [83] and paclitaxel [84] through HIF-1-mediated G0/G1 arrest. Silencing endogenous HIF-1α by RNA interference switches G0/G1 arrest into the cell cycle, ultimately, recovers the responsiveness of cells to paclitaxel, thereby establishing the central role of HIF-1α in hypoxia-induced chemoresistance.

Pretreatment with an activator of ERK induced nuclear translocation of activated ERK2, which led to the suppression of cisplatin-induced apoptosis. This indicates that pre-localization of activated ERK2 in the nuclei contribute to cisplatin resistance [85]. Inhibition of PI3K with LY294002, a flavonoid derivative which is a competitive and reversible inhibitor of the ATP-binding site of PI3K, sensitized ovarian cancer cells to carboplatin [86]. Selective inhibition of PI3K decreased growth of ovarian carcinoma and ascites formation in an athymic mouse xenogenetic transplant model of ovarian cancer [87].

Moreover, there seems to be a role for the soluble form of IL-6R in chemoresistance as well; IL-6 trans-signaling on endothelial cells could provide anti-apoptotic signals, helping endothelial cells to escape drug-mediated destruction and thus decreasing the efficacy of chemotherapy [41].

IL-6 involvement in anti-cancer immunity. As mentioned before, IL-6 can create a highly immunosuppressive environment. Macrophages are usually the most abundant immune population present in the tumor microenvironment. They can come in two flavors (M1 and M2) of which the latter have poor antigen-presenting capacity, prevent T-cell activation, and may contribute to suppressing DC functions [17]. In ovarian cancer, TAM density is correlated with a poor prognosis [88]. Macrophages in the ascites of advanced epithelial ovarian cancer patients are polarized to M2 macrophages stimulated by cancer-derived factors including IL-6 [88-90].

IL-6 in combination with macrophage chemokine monocyte chemoattractant protein-1 (MCP-1; CCL2), which is abundantly present in ovarian cancer [91], promotes an immunosuppressive environment by recruitment of monocytes to the tumor microenvironment and their differentiation towards the immunosuppressive and tumor promoting M2-type of macrophages instead of fully matured DC required to mount the appropriate tumor immune response [92]. IL-6 induced Stat3 mediates tumor-induced immunosuppression at many levels. Stat3 is one of the major regulators of macrophage activation and is associated with polarization towards M2 macrophages [93]. Stat3 activation was involved in cell-to-cell interaction between macrophages and cancer cells and occurs via soluble factors including

IL-6. Interestingly, Stat3 activation was also detected in cancer cells by co-culturing with macrophages, and blockade of Stat3 activation in macrophages suppressed Stat3 activation in cancer cells [93]. These results indicate that cancer cell survival and proliferation in the peritoneal microenvironment are significantly influenced by macrophage differentiation and activation towards the M2 phenotype via Stat3 signaling [93]. Stat3 phosphorylation can promote Treg proliferation within the tumor [44].

Another involvement of IL-6 in creating a highly immunosuppressive environment is by stimulating B7-H4 expression. B7-H4 belongs to the B7 family, which consists of activating and inhibitory co-stimulatory molecules that positively and negatively regulate immune responses. B7-H4 is responsible for negative signals that control and suppress T-cell responses. High levels of B7-H4 protein are found in ovarian cancers [94] as well as on a population of TAMs in patients with ovarian carcinoma [95]. It was shown that Tregs and B7-H4+ macrophages are co-localized in the tumor tissue and that their numbers are correlated [8]. Tregs trigger the production of high levels of IL-6 and IL-10 production by APCs and in turn, these cytokines stimulate the expression of B7-H4 by APCs, which renders the APCs immunosuppressive [95].

The IL-6 induced survival proteins, proliferation proteins and pro-angiogenic factors also influence the immune system. In addition to cancer cells, macrophages can upregulate pro-angiogenic factors as MMP-2 and -9, contributing to angiogenesis and metastasis as well [44].

Regulation of T cell apoptosis plays a critical role in controlling immune responses and XIAP is a key regulator of apoptosis. XIAP can regulate T cell effector function. By inhibiting caspase-activity and downregulation of NFκB and FasL cancer cells can have a direct escape from CD8+ T cell attack [72;96;97].

IL-6 AS A THERAPEUTIC TARGET

Therapeutic targeted therapy of IL-6 and its receptor in ovarian cancer has a strong biologic rationale. In this part we discuss the preclinical and clinical findings to target the IL-6/IL-6R pathway. Since this is a relatively new field within the treatment of ovarian cancer, only a few studies have been performed. Therefore, we will also discuss the results of the clinical trials targeting the IL-6(R) in other solid tumors such as renal cell and prostate cancer. In these types of solid cancers, high levels of IL-6 and sIL-6R in sera of patients are correlated with a poor prognosis as well.

Preclinical evidence

Targeting IL-6. In ovarian cancer, there is only one study that investigated the effect of blocking IL-6 with a monoclonal antibody [98], CNTO328. CNTO328 or siltuximab, is a chime-

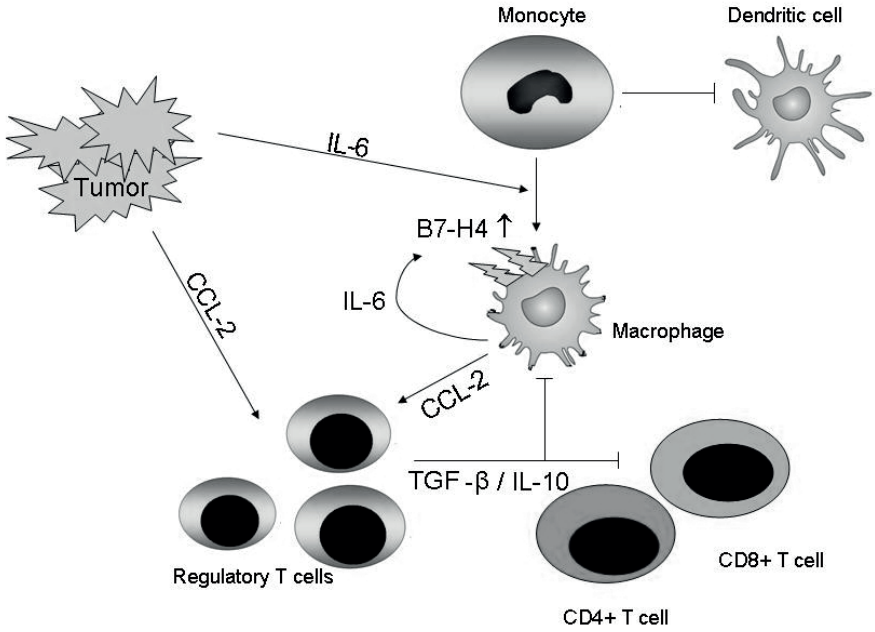

Figure 4. Schematic model of the tumor micro-environment in ovarian cancer.
Tumor cells polarize – by secreting IL-6 – monocytes into macrophages, which in turn produce IL-6/IL-10. Tumor cells are the major source of CCL-2. Tregs are attracted to the microenvironment by CCL-2, which is first secreted by the tumor and later by the TAMs. Tregs mediate their inhibitory activities by producing immune suppressive cytokines (eg TGF-β and IL-10) and can trigger IL-6 production by APCs. IL-6 stimulates B7-H4 expression on macrophages and B7-H4 loaded macrophages cause cell cycle arrest.

rized mouse mAb to IL-6 (human-mouse cmAb to IL-6). In this study [98], siltuximab inhibited IL-6 induced Stat3 phosphorylation in paclitaxel-resistant ovarian cancer cells. When ovarian cancer cells were incubated with either IL-6 alone or with siltuximab, a significantly reduced pStat3 expression in the cell lines treated with siltuximab was observed. Siltuximab also inhibited Stat3-mediated anti-apoptotic protein expression and nucleocytoplasmic translocation of Stat3, thereby resulting in an increased paclitaxel sensitivity in paclitaxel-resistant ovarian cancer cells in vitro as measured by MTT cytotoxic assay. In vivo, using a xenograft mouse model, a combination of siltuximab (20 mg/kg) and paclitaxel (20mg/kg) did not show a significant effect on tumor growth. The dosage of both drugs might not be suitable for mice or siltuximab might not have penetrated into the tumor site.

Alterations in the immune system are also found when blocking IL-6. By inhibiting IL-6 and IL-10 signaling by neutralizing antibodies (Pepro Thech), as they were thought to induce this Stat3 activation. Stat3 activation was significantly suppressed, but the inhibition level was 40% [93]. This suggests that there might be other unknown molecules involved. M-CSF, VEGF and prostanoids (eg PgE2) are good candidates, since these molecules are known to activate Stat3 signals and there production is enhanced in the co-culture of macrophages

and epithelial ovarian cancer cells. Another explanation might be that Stat3 is still activated through the soluble forms.

Targeting sIL-6R. Lo *et al*. [41] hypothesized that the combination of IL-6 and sIL-6R present in the tumor microenvironment facilitates IL-6 trans-signaling. To test this hypothesis, they studied the effects of a Fc-Ab targeting only the soluble gp130 protein of the IL-6R [sgp130Fc] – which selectively inhibits the soluble form of IL-6R – on the progression of intraperitoneal tumors. Mice were treated weekly with phosphate buffered saline (PBS) or sgp130Fc (100 μg/mouse), alone or in combination with paclitaxel (10 mg/kg). All PBS-treated animals displayed increasing intraperitoneal tumor burden and signs of ascites formation following tumor inoculation. Significant tumor suppression by paclitaxel was observed 3 weeks after initiation of treatment. The mean tumor burden in the paclitaxel-treated group (2.2×10^9 photons/s) was reduced by approximately 67% compared with the control group (6.7×10^9 photons/s) at 42 days after injection. Sgp130Fc alone only displayed modest inhibition of tumor growth. Notably, the combination of sgp130Fc with paclitaxel markedly reduced tumor burden (2.6×10^8 photons/s) compared with paclitaxel alone ($P < 0.05$). Treatment with single-agent sgp130Fc substantially inhibited ascites formation compared with the control group ($P = 0.02$), and treatment with sgp130Fc/Taxol combination completely inhibited measurable ascites ($P = 0.004$ versus sgp130Fc alone; $P = 0.01$ versus Taxol alone).

Studies that investigated the effect of blocking a component of the IL-6 induced signaling pathways are outlined below.

Targeting Stat3. Stat3 can be blocked with methyl-2-cyano-3,12 dioxoolean-1,9 diene-28-oate (CDDO-Me) [99], a synthetic triterpenoid. When CDDO-Me is applied to macrophages at low concentrations, it displays a variety of anti-inflammatory effects; at higher concentrations the compound inhibits cancer cell growth and proliferation. CDDO-Me induced apoptosis is associated with activation of caspase-3 and 8, cytochrome C, SOCS-1 and the inhibition of NFκB and VEGF. CDDO-Me inhibited IL-6 secretion and phosphorylation and consequent Stat3 nuclear translocation in ovarian cancer cell lines, including drug resistant lines. CDDO-Me down-regulated the anti-apoptotic proteins Bcl-XL and Bcl-2 in paclitaxel resistant cell lines.

Curcumin or diferuloylmethane, commonly known as turmeric, is widely used in Asian cuisine. It is widely recognized for its anti-inflammatory, anti-microbial, and wound healing activities. Several publications [100-102] have shown that curcumin suppresses Stat-3. In ovarian cancer cells, curcumin suppresses Jak-Stat signaling via activation of PIAS-3 (the negative feedback system of IL-6), thus attenuating Stat-3 phosphorylation and tumor cell growth [103].

Blocking Stat3 can also be done with small interfering RNA, which reduced ovarian cancer cell lines to proliferate and induce cell apoptosis. It inhibited he downstream mediators as well, cyclin D, survivin and VEGF [104].

Clinical evidence

Coward *et al*. [105] recently published the first clinical study of anti-IL-6 therapy in ovarian cancer. They showed that siltuximab, when given as a single agent, has some clinical activity in recurrent, platinum-resistant ovarian cancer. Siltuximab was administered in 18 patients with advanced, platinum-resistant ovarian cancer (5.4 mg/kg every 2 weeks) in a single arm phase II trial and was well tolerated. One patient had a partial response (PR), which was accompanied by a reduction in [^{18}F]FDG uptake as detected by PET/CT imaging. In 8 patients, stable disease (SD) was achieved, lasting 6 months or more in 4 patients. Disease progressed (PD) in 10 patients. As demonstrated for other immunotherapy interventions [106], the effect on the tumor in this study might be delayed.

In other solid tumors, clinical experience with IL-6 inhibition is limited to phase I/II trials with siltuximab in renal cell and prostate cancer. The first-in-human, phase I/II study with siltuximab was conducted in 68 patients with metastatic renal cell cancer (RCC) [107]. Siltuximab was well tolerated and demonstrated a consistant safety profile. The most frequent adverse events were fatigue, chest pain, back pain, dyspnoea and hypertension. Siltuximab stabilized disease in > 50% of progressive metastatic RCC patients. One partial response was observed. Given the favorable safety profile, further evaluation of the best dosage and/or combination therapy were proposed [107]. Two studies have been performed with siltuximab in patients with prostate cancer. In the first phase 1 study [108], twenty patients who where scheduled to undergo radical prostatectomy received either no drug or siltuximab (6 mg/kg, five patients per group with administration once, two times, and three times prior to surgery). No adverse events related to siltuximab were observed. Expression of elements of the IL-6 signaling pathways was analyzed in tumor tissue by immunohistochemistry. Siltuximab treatment caused significant changes in expression of several key members of the IL-6 signaling pathway; genes immediately downstream of IL-6 in the IL-6 signaling pathway, JAK and RAS, were down-regulated by siltuximab ($P < 0.05$). The intensity of Stat3 phosphorolysation was reduced. In the second study [109], phase II, 53 patients with castration-resistant prostate cancer previously treated with one prior chemotherapeutic agent were treated with siltuximab (6mg/kg) every week for 12 cycles. Two patients (3.8%; 95% CI, 0.5-13.0%) had prostate specific antigen (PSA) response. Stable disease was measured in 7 patients (23%). The median survival of 11.6 months (95% CI, 7.5-19) in this group does not suggest an improvement over that which has been achieved with chemotherapy in this setting. Grade 4 toxicity included one case of disseminated intravascular coagulation and one episode of cerebral ischemia. Grade 3 toxicities included elevated aspartate aminotransferase/alanine aminotransferase (n=1), gastritis/esophagitis (n=2), thrombocytopenia (n=2), pain (n=2), leukopenia (n=1), and neuropathy (n=2). Further studies needs to be done to evaluate combination therapy and dose-escalation strategies.

CONCLUDING REMARKS

It is 'old news' that IL-6 has several influences in ovarian cancer. However, with an increased knowledge on the role of the IL-6/IL-6R induced pathways on proliferation, chemoresistance and on the interaction of cancer cells and the immune system, IL-6 represents a potential new target in the treatment of ovarian cancer.

Recently, it has been shown that IL-6 induced inflammatory cytokine production, tumor angiogenesis and tumor macrophage infiltration in ovarian cancer can be inhibited by a neutralizing antibody, siltuximab [105]. These data deserve further study.

Although in vitro data suggest that siltuximab inhibits the IL-6 induced pathways, this was not observed in mice treated with siltuximab and paclitaxel [98]. This might be due to the important fact that not only the membrane bound form of IL-6R but also the soluble part can activate the several pathways. Just recently, high levels of sIL-6R in malignant ascites of ovarian cancer patients were found to correlate with poor prognosis [41]. Blocking both the membrane bound and soluble form of IL-6(R), might be a more effective way by which the IL-6 induced pathways can be inhibited. Blocking of the cytokine IL-6 itself can be very difficult, given the high concentrations of IL-6 in patients with ovarian cancer. Blocking the receptor might overcome this problem. Tocilizumab, a humanized antihuman IL-6R antibody, recognizes both the membrane bound and soluble form of IL-6R and inhibits IL-6 signaling by preventing ligand-receptor binding and activation of gp130 [110]. It has been registered for the treatment of Castlemans disease, a lymphoproliferative disorder driven by IL-6 and rheumatoid arthritis. A phase I/II trial of carboplatin and pegylated liposomal doxorubicin with tocilizumab in patients with recurrent platinum sensitive ovarian cancer is ongoing at our hospital (www.clinicaltrials.gov).

Additionally, it remains to be determined whether multi-targeted agents or combinations of agents targeting different pathways will offer greater clinical benefit then targeting the IL-6(R) induced pathways alone. These pathways can also be activated through IL-6 independent mechanisms. The growth factor receptors that are known to activate Stat3 include the epidermal growth factor receptors EGFR and HER2 (also known as NEU), FGFR (fibroblast growth factor receptor), IGFR (insulin-like growth factor receptor), HGFR (hepatocyte growth factor receptor; also known as MET), PDGFR (platelet-derived growth factor receptor) and VEGFR (VEGF receptor). The oncoproteins SRC and ABL are also activators of Stat3 [44]. One might overcome this problem by inhibiting these pathways themselves. Preclinical evidence for blocking the Stat3 pathway is discussed before [*see IL-6 as a therapeutic target - Preclinical evidence*]. In ovarian cancer, multiple targeted agents, including sorafenib, sunitinib, imatinib, pazopanib, and cabozantinib [111-114] have been clinically tested. Three studies with sorafenib were performed, of which two [115;116] had to be stopped early due to substantial toxicity. The third study [117] combined administration of gemcitabine and sorafenib was given in patients with platinum resistant ovarium cancer. Of all 43 patients, 2

patients had a partial response, 10 patients (23.3%) maintained stable disease for at least 6 months. Hematologic toxicity was common, but manageable. No clinical data are yet available for Stat3 and P13K inhibitors in ovarian cancer patients. Combining all IL-6 induced pathways by blocking these pathways separately seems – for now – not a suitable option. Besides this practical comment, one might wonder what the effect of these therapy combinations is on the various cellular components of the immune system. What will they do with TAMs, considered their contribution to tumor growth? Does this influence the balance between immunosuppressive and -activating stimuli? These are all questions that require more research.

The combination of chemotherapeutic agents – to lower tumor burden and to enhance the uptake of tumor antigen by local APC, a process described as immunogenic cell death [10] – with anti (s)IL-6(R) antibodies – to block IL-6 signaling leading to inhibited tumor proliferation, angiogenesis and chemoresistance and to prevent differentiation of local APC to tumor-promoting macrophages – might improve the clinical outcome of ovarian cancer patients. Preclinical and clinical studies to test this hypothesis are currently ongoing.

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