Immune-based therapies in ovarian cancer

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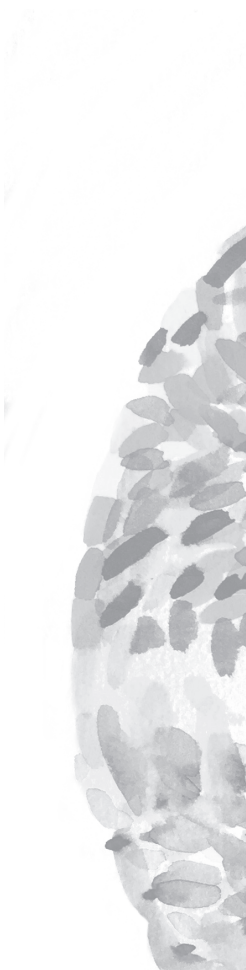
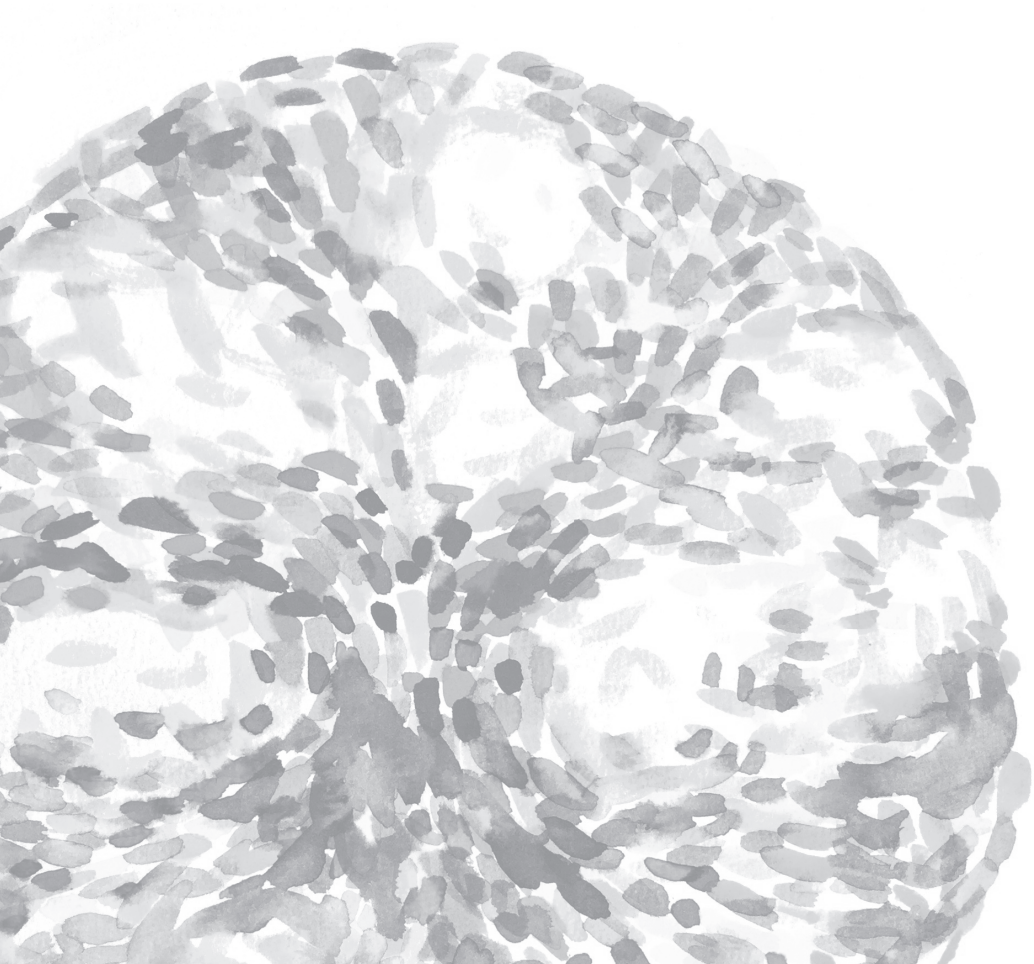


CHAPTER 1

Introduction



In this thesis, pre-clinical and clinical studies focusing on immunological aspects of ovarian cancer, and the role of chemotherapy and chemo-immunotherapy on local immunity in ovarian cancer are discussed. Here I provide an overview of the general aspects of ovarian cancer, followed by a short description of tumour-immunology and its role in ovarian cancer in particular. Finally, an outline of the thesis is provided.

OVARIAN CANCER

Ovarian cancer remains a challenging disease for which improved treatments are urgently needed as each year around 1300 new cases are diagnosed in The Netherlands leading to 1000 deaths per year [1]. Theoretically, early detection could improve outcomes, but this remains a challenging problem due to the relative rarity of the disease in the general population. The current paucity of highly sensitive and specific biomarkers, and the idea that the disease develops and spreads exceptionally rapidly, leaves only a small window of opportunity for early detection [2]. By virtue of their anatomical location, ovarian tumours frequently disseminate throughout the peritoneal cavity and implant on various abdominal organs, while also inducing ascites in the abdominal cavity in many cases. The degree of tumour dissemination is reflected in the International Federation of Gynecology and Obstetrics (FIGO) staging system: stage I–II disease is confined to one or both ovaries, stage III disease shows peritoneal spread, and stage IV disease shows distant metastases [3]. Ovarian cancer can be divided into three broad subgroups – epithelial, stromal and germ cell tumours – each with different aetiologies and clinical behaviour [4;5]. Epithelial ovarian cancer (EOC) is the most common type – constituting more than 85% of all cases of ovarian cancer – and is therefore the focus of this thesis.

CURRENT TREATMENT

Standard care for advanced EOC (stage IIB-IV) is maximal cytoreductive surgery followed by chemotherapy with platinum-based agents (mostly carboplatin) in combination with taxanes (paclitaxel). Several clinical features are examined in order to determine which available therapies would be most effective in yielding a favourable outcome. For instance, assessment of histologic subtype, tumour grade, stage, age, and the size of the residual tumour after primary cytoreductive surgery allows physicians to make informed decisions regarding the application of anticancer strategies [6-8]. Increased knowledge on the heterogeneity of the disease has led to the identification of distinct molecular signatures for the different histologic subtypes, providing potential targets for therapy, such as p53, breast cancer (BRCA) and retinoblastoma 1 (RB1) [9-11]. Despite the activity of chemotherapy, which gives

response rates up to 80% in first-line treatment, resistance to chemotherapy often occurs, prohibiting further curative therapy. This leads to a median progression free survival of only 18 months and a 5 year overall survival of 35% [12]. Therefore, a large proportion of patients become candidates for second-line treatment. Platinum sensitivity, which is defined by the duration of the response to platinum-based therapy, has been found to predict the response to subsequent salvage therapy with a platinum-containing regimen. Patients who relapse within 6 months after cisplatin or carboplatin based chemotherapy are considered 'platinum-resistant', and patients who relapse later than 6 months after completion of the initial therapy are characterized as 'platinum-sensitive' [13]. Patients with platinum-resistant disease are amenable for novel investigational approaches and studies of drug resistance [14]. Both single-agent and combination chemotherapy with or without angiogenesis inhibition are considered as treatment options in these patients. Modest benefit is observed with the use of agents like paclitaxel, pegylated liposomal doxorubicin (PLD), gemcitabine, topotecan and oral etoposide [15]. Recently, the anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab has been registered for both platinum sensitive and platinum resistant disease [16]. In the Netherlands it is used in patients with platinum sensitive disease as second line treatment in combination with carboplatin and gemcitabin. Moreover, poly ADP-ribose polymerase (PARP) inhibitors are a novel type of medication that works by preventing cancer cells from base excision repair and which results in synthetic lethality in homologous recombination deficient deficient tumours. The PARP inhibitor olaparib improves progression free survival in women with recurrent platinum-sensitive disease with the greatest clinical benefit in BRCA-mutated patients [17]. Olaparib has been approved for the maintenance treatment of adult patients with platinum sensitive relapsed BRCA mutated high grade serous EOC after response to platinum based therapy.

IMMUNITY IN OVARIAN CANCER

In the 1890s, Williams Coley, a surgeon in New York, demonstrated that injection of streptococcal bacteria into the inoperable tumours of cancer patients could stimulate the immune response to combat tumours. Nowadays, cancer immunotherapy is a promising and effective treatment modality for patients with various immunogenic cancer types. Increasing evidence supports the idea that ovarian cancer also is an immunogenic tumour since the prognosis of EOC patients is associated with the density of tumour infiltrating immune effector cells. Hence, an immunotherapeutic approach to treat ovarian cancer seems promising.

In the immune response to cancer, several actors play different roles during three distinctive phases in tumour development and progression: elimination, equilibrium and escape [18]. The ensemble of these events is called immunoediting (**Figure 1**). T cells are the principal actors of the first two phases (elimination and equilibrium) in which the im-

mune system recognizes and eliminates cancer cells or control their growth with as result complete control of the tumour. The cancer becomes clinically relevant when, despite of these mechanisms, its cells acquire the capacity of either escaping or suppressing the patient's immune system. The phase of *elimination* starts when growth of cancer tissues induces the release of immune cell attracting chemokines ad cytokines allowing elements of the innate compartment (Natural Killer cells (NKs), γδ T-cells, dendritic cells (DCs) and M1-macrophages) to interact with the tumour. The resulting production of interferon gamma (IFN-γ) and interleukin 12 (IL-12), with a positive feedback, leads to control of tumour growth by suppression of its proliferation and neo-angiogenesis and by promoting the tumour cells to undergo apoptosis [18-21]. In addition, it provides the start for the adaptive immunity. After the ingestion of cancer cells' components, the DCs - activated by tumour-released danger signals, cytokines or directly by NKs - migrate to local lymph nodes, where they activate cancer-specific T-helper type 1 (Th1; CD4+) and CD8+ cytotoxic T lymphocytes (CTLs). In cancer, IFN-γ producing Th1 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. Tumour-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 tumours and to exert their effector function against the cancer cells [22]. In the case of ovarian cancer, a dense infiltration with intratumoural CD8+ T cells correlates with improved progression free survival and overall survival [23]. In addition, CD3+ CD56+ cells, containing the NK-like T cytotoxic cells, play a role in this phase and their presence in the ascitic fluid in ovarian cancer patients seems to correlate with a better prognosis [24].

The cancer cells that are not eliminated in this phase may then enter the *equilibrium* phase, in which further tumour development is prevented by adaptive immunologic mechanism. CD8+ T cells and DCs secrete INF-γ and IL-12, respectively, and keep tumour growth at bay. In this period several tumour cell variants may arise but are still under immune control. This dynamic balance can persist for long period, sometimes exceeding 20 years [25]. *Escape* is the third phase in which cancer cells finally succeeded to evade the immune system. During this phase, the immune system fails to restrict tumour outgrowth and tumour nodules emerge causing clinically apparent disease. In this phase, tumour cells evade immune recognition (by loss of tumour antigens, major histocompatibility complex (MHC) class I or co-stimulatory molecules), express molecules of increased resistance (signal transducer and activator of transcription 3 (STAT-3)), survival (anti-apoptotic molecule bcl2) and immunosuppression (indoleamine 2, 3-dioxygenase (IDO), tryptophan 2,3-dioxygenase (TDO), programmed death-ligand 1 (PD-L1), galectin-1/3/9, CD39, CD73, adenosine receptors) and secrete cytokines (VEGF, transforming growth factor beta (TGF-β), IL-6, macrophage colony stimulating factor (M-CSF)) that enhance angiogenesis and modulate the immune microenvironment within the tumour [20].

The myeloid arm of the immune system can give rise to multiple types of cells, including macrophages, DCs, neutrophils and myeloid-derived suppressor cells (MDSCs). Macrophages that infiltrate cancer tissue are often referred to as tumour-associated macrophages (TAM). Macrophages can differentiate from monocytes into tumour-rejecting M1 or tumour-promoting M2 macrophages depending on the environmental cues they encounter. M1 macrophages produce IL-12 needed to stimulate anti-tumour immunity and have the potential to kill tumour cells. They can ingest dead (necrotic) tumour cells in order to present tumour (associated) antigens to the infiltrated T cells, which subsequently can kill other tumour cells, resulting in inhibition of tumour growth or regression [26;27]. This type of cell is more often found during the elimination phase of tumours. However, tumour cells and some myeloid cells can produce large amounts of immunosuppressive cytokines (e.g. TGF-β and IL-10) and soluble mediators, *e.g.* prostanoids such as prostaglandin E2 (PgE2) and IL-6 [28]. These products lead to the development of M2 macrophages, which produce amongst others IL-10 (and no IL-12), suppress adaptive immunity and promote matrix remodelling, tumour growth, invasion and metastasis as well as promote angiogenesis.

Moreover, they can lead to tumour-associated MDSCs, which are known to expand dramatically in advanced cancer [29] and can suppress anti-tumour responses through several mechanisms: suppression of $CD4^+$ and $CD8^+$ T cells by arginine and cysteine depletion, inhibition of T cell recruitment to tumor sites, inhibition of T cell-peptide-MHC interactions, skewing of the cytokine milieu toward type 2 or regulatory responses, and modulating NK and NKT responses [29-31]. In addition to their immunosuppressive properties, MDSCs can secrete factors (*e.g.* VEGF) that enhance tumour growth, invasion, and metastasis [32]. M2 macrophages and MDSC are the predominant tumour infiltrating myeloid cell type in the escape phase. Furthermore, MDSCs, M2 macrophages and DCs may also produce immunoregulatory molecules such as arginase 1, inducible nitric oxide synthase (iNOS) and express IDO and secrete immunosuppressive cytokines IL-10 and TGF-β that can inhibit $CD8^+$ proliferation and function or induce apoptosis. MDSCs and IDO expressing DCs also induces the generation of regulatory T cells (Tregs) [33].

Tregs are a heterogeneous T cell subpopulation whose primary function is suppression of immune responses and preventing autoimmunity and immunopathogensis. Most Tregs identified so far belong to the CD4+ lineage, but CD8+ Tregs have been also reported. In ovarian cancer, CD4+FoxP3+CD25+ Tregs, a subset of T cells endowed with powerful suppressor activity, are an important mediator of peripheral immune tolerance. These cells prevent T cell-specific immunity by suppressing CD8+ T cell activation, inhibit secretion of IL-2 and IFN-γ and help other immunosuppressive populations like tolerogenic APCs [34-36]. Tregs may express inhibitory receptors such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and lymphocyte activation gene 3 (LAG3) that suppresses anti-tumour immune response and favour tumour outgrowth [37].

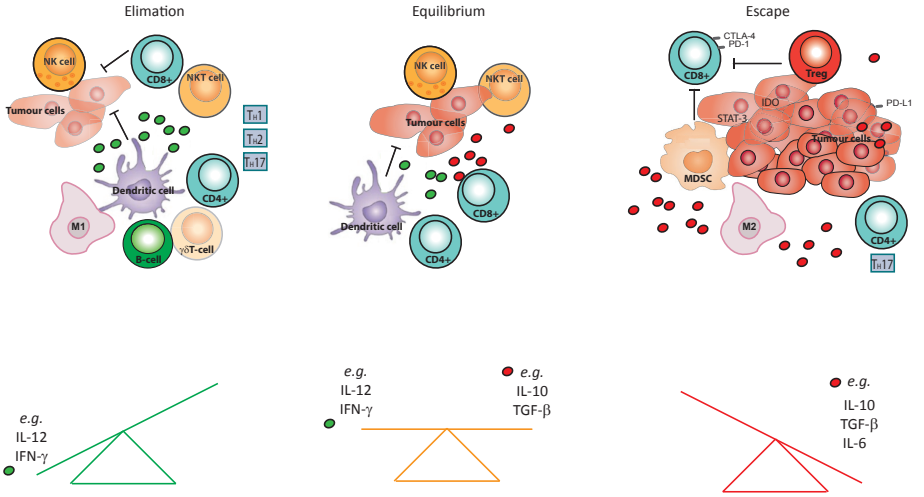


Figure 1. Immunoediting.
Elimination is a phase of cancer immunoediting where both the innate and adaptive immune system together detect and destroy early tumours before they become clinically visible. In the **Equilibrium** phase of cancer immunoediting, the immune system holds the tumour in a state of functional dormancy. Some tumour cells undergo genetic and epigenetic changes and due to constant immune pressure, tumour cell variants evolve that resist immune recognition (antigen loss or defects in antigen presentation) and induce immunosuppression (PD-L1). The Equilibrium phase is a balance between anti-tumour (IL-12, IFN-γ) and tumour promoting cytokines (IL-10, IL-23). The adaptive immune system is required to maintain tumour in a functionally dormant state while NK cells and cytokines such as IL-4, IL-17A and IFN-α/β are dispensable. During the **Escape** phase of cancer immunoediting, the immune system fails to restrict tumour outgrowth and tumour cells emerge causing clinically apparent disease. In this phase, tumour cells evade immune recognition (loss of tumour antigens, MHC class I or co-stimulatory molecules), express molecules of increased resistance (STAT-3), survival (anti-apoptotic molecule bcl2) and immunosuppression (IDO, TDO, PD-L1, galectin-1/3/9, CD39, CD73, adenosine receptors) and secrete cytokines VEGF, TGF-β, IL-6, M-CSF that enhance angiogenesis. Adapted from [38].

IMMUNOTHERAPY IN OVARIAN CANCER

Knowledge of the tumour immune microenvironment helps to deduce the requirements for good tumour control and subsequently effective tumour immunotherapy in patients with ovarian cancer. The first goal is to control tumour growth to earn the time needed for an effective immune response to develop. Activation of professional APCs by engaging costimulatory receptors is the first step. Secondly, tumour-specific T cells should be expanded, which may occur spontaneously but can also be boosted by vaccines or immune stimulatory monoclonal antibodies. Finally, these T cells need to arrive at the site of action which is the tumour, but the tumour microenvironment should be optimized to support their effector function.

Current proven and experimental immunotherapies for ovarian cancer can be divided in two broad categories: passive and active immune therapy.

The infusion of *monoclonal antibodies* that target specific molecules expressed by tumour cells or in the tumour microenvironment with the aim to block growth signals in tumour cells are forms of passive immunotherapy because this therapy does not use the immune system of the patient. An example is bevacizumab, which targets VEGF, and is the first approved immunotherapeutic drug in ovarian cancer [39].

Active immune therapies stimulate the patient's own immune system to attack their tumour. There are many different therapies in development, of which checkpoint inhibitors, immune modulators, therapeutic vaccines, adoptive T cell transfer, and oncolytic viruses are well-known. One promising avenue of clinical research in ovarian cancer is the use of *antibodies targeting inhibitory molecules* (i.e. checkpoint blockers), that serve as checks and balances in the regulation of immune responses. By blocking inhibitory molecules expressed by activated T cells or, alternatively, by activating co-stimulatory molecules, these treatments are designed to remove the breaks and activate pre-existing anti-cancer T cell responses. Clinical studies in other malignancies have already shown clinical efficacy of this type of immunotherapy. In patients with epithelial ovarian cancer the efficacy of PD(L)-1 [40;41] and CTLA-4 antibody [42] are investigated.

The fact that both the presence of tumour infiltrating lymphocytes (TILs) correlated strongly with survival and that 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. This is a technique using autologous or allogeneic antitumour lymphocytes to induce cancer regression. There are many forms of adoptive T cell therapy being used for cancer treatment; culturing tumour infiltrating lymphocytes or TIL, isolating and expanding one particular type of T cell or clone, and even using T cells that have been engineered to potently recognize and attack tumours. In ovarian cancer, however, only three phase I trials have been conducted so far, with contradictory results [43-45]. Therefore, more and extended phase II trials are required to investigate whether this is an effective method in ovarian cancer patients, as seen for other malignancies [46-48].

Therapeutic *vaccine therapies* are based on the existence of ovarian cancer-associated antigens—molecules on or in cells that can serve as targets for immune recognition and attack. These include several "cancer-testis" antigens, which are expressed only by cancer cells and not by healthy tissues (with the exception of the testis and, occasionally, placenta), making those promising targets for cancer immunotherapy. Examples of cancer associated antigens are cancer antigen 125 (CA125), p53, Wilms tumour protein 1 (WT-1) and New York Esophageal Squamous Cell Carcinoma 1 (NY-ESO-1) [34]. As persistent overexpression of p53 or induced T-cell presentation is present in ~50% of a wide variety of cancers among which ovarian cancer [49], a large group of patients would benefit from p53 directed immunotherapy. Two phase I/II clinical trials using p53 immunogens have been performed in

ovarian cancer patients thus far, which proved to induce antigen-specific T cell responses [50;51].

Recent studies indicate that most of the clinical benefit of checkpoint blockade and adoptive T cell transfer is mediated via T cells that specifically recognize mutated proteins present in tumours [52]. Therefore, new vaccines and cell transfer strategies may alternatively focus on mutated antigens in ovarian cancer. Finally, *oncolytic virus therapy* using a modified virus that specifically cause tumour cell destruction and induces local inflammation may also be used to attract and stimulate tumour-specific immune responses to tumour antigens released from the killed cancer cells.

INFLUENCE OF CHEMOTHERAPY ON IMMUNITY

In the past years, it has become clear that interference with only one element of the immune system will not suffice to eradicate cancer in the majority of patients. Effective immunotherapy will have to combine immune-activating strategies with elimination of immune-suppressing mechanisms. In this respect, it is interesting to know that evidence is accumulating suggesting that the immune system plays a role in the efficacy of chemotherapy as well. Although the main mechanism of action of chemotherapy is to kill fast proliferating tumour cells, recent articles suggest that chemotherapy also rely on serval off-target effects, especially directed to the host immune system, that cooperate for successful tumour eradication [53]. For instance, if cancer patients suffer from lymphopenia before start of treatment, they are less likely to respond to chemotherapy suggesting that immune cells are important for the response to chemotherapy [54].

Conventional chemotherapy can activate anticancer immune responses through different mechanisms; these mechanisms include activation of tumour-specific immunity, conversion of the tumour milieu into a site permissive for T cells via mitigation of immune suppression, enhancement of antigen delivery to tumour-associated DCs, increased T-cell infiltration and sensitization of cancer cells for T-cell mediated cytotoxicity [55]. Paclitaxel, gemcitabine and oxalipatin can cause immunogenic cell death, leading to promotion of tumour expression of ecto-calreticulin, and release of high mobility group box 1 (HMGB-1) and adenosine triphosphate (ATP) by dying tumour cells, thus stimulating antigen phagocytosis and cross-presentation by DCs [56]. Paclitaxel induces pro-inflammatory cytokine secretion from macrophages, leading to DC, NK and T cell activation [53]. Gemcitabine depletes Tregs or MDSCs and facilitates tumour attack by effectors *in mice* [29;57]. Notably, most of these studies were performed *in vitro* or *in vivo* using mouse-models. The combination of chemotherapeutic agents with immunotherapy might prove a strategy to improve the clinical outcome of ovarian cancer patients. However, the effect of chemotherapy on immune cells in humans remains largely unclear.

OUTLINE OF THE THESIS

The generally poor prognosis of patients with epithelial ovarian cancer (EOC) patients treated with curative intent, calls for additional treatment modalities and possible success might lie in a combination of chemotherapy and immunotherapy. This thesis focuses on the interaction of chemotherapy with the immune system and describes new combined chemo-immunotherapy treatment strategies.

IL-6 is a major mediator of cancer-related inflammation by stimulating inflammatory cytokine production, tumour growth, tumour angiogenesis, and tumour macrophage infiltration. High levels in sera and ascites of ovarian cancer patients have been associated with a worse prognosis [58;59]. In **Chapter 2**, the role of IL-6 in EOC is reviewed and its expression, regulation and function in the tumour immunity is detailed. The current and future possibilities of blockade of the IL-6/IL-6R pathway in ovarian cancer are discussed.

In **Chapter 3,** the immune-modulating property of chemotherapy on tumour cells and different immune cells in the tumour microenvironment was investigated. These studies revealed that chemotherapy enhanced IL-6 release by tumour cells and the conversion of monocytes to M2 macrophages as a consequence. Subsequently, the use of different immune-modulating agents to circumvent this unwanted effect was investigated.

In **Chapter 4**, the intricate interaction between the IL-6 signalling pathway and tumour-infiltrating myeloid cells as well as their prognostic impact in EOC was studied. The expression of IL-6, IL-6 Receptor (IL-6R) and phosphorylated signal transducer and activator of transcription 3 (pSTAT3) as well as the number and type of infiltrating myeloid cells present in EOC was studied. In addition, the relationship between these markers, tumour-infiltrating immune cells, clinical-pathological characteristics and their prognostic or therapeutic impact in a cohort of patients with EOC was determined. A clear relation between tumour-expressed IL-6, a higher number of M2 macrophages and a shorter survival was found.

In a first attempt to change the IL-6 associated immune suppressive myeloid cell composition, we initiated the PITCH trial (**Chapter 5**). In this phase I trial standard chemotherapy, carboplatin and (pegylated liposomal) doxorubicin, is combined with tocilizumab, a monoclonal antibody against IL-6R, with and without Peg-Intron (interferon 2α-b), in patients with platinum sensitive recurrent EOC. The feasibility and the effect on the immune system of this new treatment regimen is studied.

In **Chapter 6** a different strategy was employed to not only modulate immune suppressive myeloid cells but also to activate T cells against the cancer associated antigen p53. Manipulation of myeloid cells was performed using gemcitabine and interferon alpha. Gemcitabine has a significant immune-stimulatory activity in addition to its direct cytotoxic effect in platinum resistant ovarian cancer and is therefore an attractive agent for the combination with immunotherapy. Interferon alpha has multiple immune stimulatory effects, including the upregulation of HLA molecules, increased antigen processing and enhancement

of activated T cell survival. Therefore, in patients with platinum resistant ovarian cancer, a new treatment combination of gemcitabine, Peg-Intron with or without p53 synthetic long peptide (SLP) vaccination was studied.

In **Chapter 7**, a general discussion of the reported studies described in this thesis is discussed within a broader view of recent literature and future perspectives.

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