Chapter 1

Introduction to the thesis
Ovarian cancer: epidemiology, diagnosis, and treatment

Epithelial ovarian cancer is the deadliest gynecological malignancy. Yearly, approximately 1100 women are diagnosed in the Netherlands, 900 of which eventually succumb to the disease (1). Ovarian cancers are histologically classified as serous, mucinous, clear cell, endometrioid, transitional (Brenner), or squamous cell tumors (2). The histopathology of these subtypes has been the subject of many recent discussions, in which extra-ovarian origins are increasingly being recognized. Serous carcinomas were traditionally thought to originate from the ovarian surface epithelium, due repeated tissue damage at ovulation, but ovarian cortical inclusion cysts and the peritoneum at large were also putative sites of origin (3). However, the identification of precursor lesions in the fallopian tubes, termed serous tubal intraepithelial carcinomas (STICs), has challenged this view and resulted in the ‘tubal hypothesis’ for serous ovarian cancers (3,4). Recently, similar precursor lesions were found in the endometrium, further expanding the list of possible sites of origin (5).

The origin of non-serous tumor types was also a topic of investigation in recent years. There is now some consensus that endometrioid and clear cell tumors can arise from atypical endometriosis lesions (6). In the case of mucinous tumors, the distinction between primary and metastatic disease is difficult. Primary mucinous ovarian cancers are now considered rare. Rather, they are often metastases from gastrointestinal tumors such as appendiceal pseudomyxoma peritonei (7). Deciphering the precise origin of ovarian cancer is not just interesting from an academic point of view, but carries important clinical consequences, because factors such as sensitivity to chemotherapy tend to differ between subtypes (3). Careful pathological examination is therefore of paramount importance.

Clinically, early detection of ovarian cancer is notoriously difficult. Ovarian cancer was historically referred to as ‘the silent lady killer’, because of its perceived lack of symptoms. In recent years it became clear that ovarian cancer is certainly associated with symptoms, but that these are quite aspecific and therefore not recognized as being indicative of a serious illness. Symptoms most predictive of ovarian cancer include persistent abdominal distension, appetite loss, postmenopausal bleeding, and early satiety (8). These symptoms usually only manifest themselves late in the disease process; hence, 70% of women are diagnosed with advanced stage ovarian cancer (1).

Debulking surgery and platinum-based chemotherapy are the mainstays of ovarian cancer treatment. Unfortunately, recurrence is a common problem, even after complete remission. In those cases, treatment options are palliative and dictated by whether or not the tumor is still platinum sensitive. Targeted agents, such as angiogenesis inhibitor bevacizumab, are currently being investigated in clinical trials (9). However, significant improvements in survival remain to be seen.
Introduction to the thesis

Basic concepts of tumor immunology

The interaction between cancer and the immune system was recognized as early as the 19th century, when William Coley reported that administering Streptococcal toxin to patients with inoperable sarcomas could result in complete tumor regression (10). Over the next century, the concept of tumor immunology was frequently disputed, until in the mid-1980s tumor immunogenicity and the presence of auto-reactive T cells were further elucidated (11).

The current paradigm of ‘cancer immunoediting’ describes the involvement of the immune system in both tumor prevention and shaping of tumor immunogenicity (12). This concept revolves around ‘the three E’s’ of Elimination, Equilibrium, and Escape. Elimination applies to the subclinical stage of disease, in which the innate and adaptive immune system combine forces to eradicate (a limited number of) malignant cells. However, certain cells may escape elimination and enter equilibrium, a steady state in which cancer cells remain viable, but are under constant immunologic pressure by the adaptive immune system and outgrowth to full-blown tumor is prevented. The inherent danger in this situation is the emergence of immune resistant cells, which enable the formation of clinically apparent disease, thus entering the phase of escape (12). In this final stage, various immunosuppressive mechanisms may be at work, some of which are discussed in this thesis. Manipulating these unfavorable microenvironmental circumstances is key to developing clinically effective immunotherapy.

Immunotherapy has already shown real promise in several types of cancer. For instance, anti-cytotoxic T lymphocyte associated antigen 4 (CTLA4) antibodies are now registered for adjuvant use in melanoma patients (13). Moreover, vaccination with human papillomavirus (HPV) peptides induced regression of high grade vulvar intraepithelial neoplasia (14). Successes like these are still elusive in ovarian cancer, but do underline the principle that modulating the immune system can change the tumor microenvironment for the better.

Recognition of tumor cells by the immune system

The immune system contains an elaborate system of checks and balances, to prevent auto-immune phenomena. In cancer, on the other hand, the immune system should recognize the patients’ own, malignantly transformed, cells. The distinction between healthy cells and tumor cells is provided by tumor antigens. When presented to naïve immune cells in the context of appropriate co-stimulation, an immune response can be mounted against these antigens and thus against the malignant cells expressing them.

Tumor antigens are traditionally divided in two classes: tumor specific antigens (TSAs) and tumor associated antigens (TAAs). TSAs are caused by gene mutations, whereas TAAs are over- or aberrantly expressed non-mutated molecules (15). TAAs are exclusively expressed on cancer cells, making them a prime target for immunotherapeutic intervention. However, because considerable inter-patient variation exists regarding their expression pattern, individualized approaches would
be needed (15).
A downside to the TAA vs. TSA classification of tumor antigens is that a certain amount of overlap exists, when antigens initially classified as TAAs turned out to be expressed on healthy cells as well. Therefore, a classification based on structure and source of the antigens is currently more in use, resulting in seven groups of tumor antigens: differentiation antigens (e.g. tyrosinase, gp-100), mutational antigens (e.g. β-catenin, caspase), amplification antigens (e.g. HER-2/neu, p53), splice variant antigens (e.g. ING1, NY-CO-37/PDZ), glycolipid antigens (e.g. MUC1), viral antigens (e.g. HPV), and cancer testis antigens (e.g. MAGE, NY-ESO-1) (16).

Lymphocyte subsets involved in the anti-tumor immune response

The immune system contains an innate and an adaptive branch. Innate immunity provides a first line of defense to pathogens. Examples of innate immune cells include neutrophils, dendritic cells, natural killer (NK) cells, and macrophages. An important characteristic of innate immunity is that it is not capable of antigen recognition, but rather responds to certain molecular patterns shared by microorganisms, or to inflammatory mediators produced by other components of the immune system. Secondly, it does not possess immunologic memory.

In terms of tumor immunology, innate immunity can contribute to the anti-tumor immune response, for instance via antigen presentation to naïve T lymphocytes or by attracting immune cells through cytokine secretion. However, innate immunity is also viewed as an instigator of carcinogenesis. This is especially seen in states of chronic inflammation, where constant tissue damage and repair ultimately results in malignant transformation (17,18). Examples of such inflammatory cancers include cervical cancer (HPV), gastric cancer (Helicobacter pylori), esophageal cancer (Barrett’s metaplasia), colorectal cancer (inflammatory bowel disease), hepatocellular cancer (hepatitis B and C), lung cancer (asbestos, cigarette smoke) and Kaposi’s sarcoma (human herpesvirus type 8) (18).

The focus of this thesis is the role of the adaptive immune system in cancer. The adaptive immune system consists of T and B lymphocytes. CD3+ T lymphocytes are key players of the anti-tumor immune response. They are divided into several subgroups, based on their surface markers and functional characteristics. CD8+ cytotoxic T lymphocytes (CTL) are the principle effector cells, killing target cells via secretion of perforins and granzymes or via Fas-ligand (FasL) induced apoptosis. CD4+ T helper (Th) lymphocytes are a heterogeneous class of cytokine secreting T lymphocytes, with both pro- and anti-tumor effects. CD4+ T helper type 1 (Th1) lymphocytes mainly secrete interferon (IFN)-γ, as opposed to T helper type 2 (Th2) lymphocytes, which secrete interleukin (IL)-4, -5, and -13. CD4+ CD25+ FoxP3+ regulatory T cells (Treg) are important in maintaining self-tolerance, i.e. preventing auto-immunity. They inhibit other T lymphocytes, but also macrophages and dendritic cells by secretion of immunosuppressive cytokines such as IL-10 or via direct cell-cell contact. The most recently recognized member of the CD4+ family is the Th17 T lymphocyte. It principally secretes IL-17, which is known to stimulate angiogenesis and neutrophil recruitment.
(19). Intriguingly, it was found that Th17 share a common precursor with a subset of Treg. Activation of naive CD4+ T lymphocytes in a Transforming Growth Factor (TGF)-β rich environment favors differentiation into Treg. When TGF-β and IL-6 are both present, the same naive CD4+ T lymphocytes tend to end up as Th17 cells (20). Thus, Th17 and Treg are essentially two sides of a coin. The role of Th17 lymphocytes in tumor immunology is not fully elucidated yet, although both pro- and anti-tumor effects have been reported (19).

After activation by antigen recognition, some effector T lymphocytes are converted to memory T lymphocytes. Memory T lymphocytes respond more efficiently than naive T lymphocytes to antigen recognition, resulting in a fast and large immune response when the antigen is encountered again. Memory T lymphocytes are subdivided based on their anatomical location, which is determined by the expression pattern of certain homing molecules. Effector memory T lymphocytes (T_{EM}) express CCR5, which is involved in homing to inflammatory sites. Hence, T_{EM} are primarily found in the circulation or in nonlymphoid tissues. They are a first line of defense against pathogens, geared towards immediate effector function. Central memory T lymphocytes (T_{CM}) lack CCR5, but express CD62L and CCR7, enabling their entry into lymph nodes via high endothelial venules (HEVs). T_{CM} selectively circulate through secondary lymphoid tissues and are precluded from entering epithelia and underlying tissues. They serve as a second line of defense, in case pathogens escape from their epithelial entry site to lymph nodes. However, even though they respond more slowly, T_{CM} have a higher proliferative capacity than T_{EM} (21).

Recent history of ovarian tumor immunology

In ovarian cancer, understanding of tumor immunology has taken great leaps forward over the past decade. A landmark paper in 2003 described how the presence of CD3+ T lymphocytes in advanced stage ovarian cancer predicts improved progression free and overall survival (22). When looking at lymphocyte subsets in more detail, the negative contribution of Treg to the anti-tumor immune response was a major finding. Curiel et al. first identified that Treg preferentially accumulate in tumor tissue and ascites of ovarian cancer patients, due to CCL22 production by tumor cells and microenvironmental macrophages (23). In their cohort of 70 ovarian cancer patients, presence of Treg was associated with reduced survival. Later studies confirmed the detrimental role of Treg, but added the notion that instead of absolute numbers of tumor infiltrating lymphocytes (TIL), their ratios compared to other immune cells determine outcome. For instance, a high CTL/Treg ratio was found to be more predictive of improved survival than high CTL or low Treg numbers on their own (24).

In recent years, another important focus of tumor immunology research in ovarian cancer was identifying which factors in the tumor microenvironment determine lymphocyte infiltration and/or function. For instance, expression of the endothelin B receptor (ETbR) on ovarian tumor endothelium was reported to be associated with fewer TIL and consequently a worse prognosis (25). These effects were mediated by downregulation of intercellular adhesion molecule-1 (ICAM-
an adhesion molecule which facilitates T lymphocyte adherence and transmigration through the endothelium. Indeed, upon *in vivo* blockade of ETβR in ovarian cancer bearing mice, ICAM-1 was upregulated and numbers of TIL increased. However, no clinical effects were observed. Ovarian cancer cells themselves can also actively inhibit lymphocytes. The pathway that probably gained the most attention in this respect is programmed cell death ligand (PD-L)-1. In a 2007 study, it was reported that PD-L1 expression in ovarian cancer specimens correlated to poorer prognosis and lower intraepithelial CTL counts (26). PD-L1 binds receptor PD-1, which is expressed on activated lymphocytes. PD-1 subsequently induces T lymphocyte anergy or apoptosis by interfering with downstream T cell receptor signaling.

**Outline of the thesis**

In this thesis, we aimed to further characterize the immunologic environment of ovarian cancer. We examined T lymphocyte infiltrates and identified relevant immune regulatory markers in a large cohort of ovarian cancer patients. Furthermore, we assessed a potential T lymphocyte activating protein *in vitro*. The ultimate aim of the research presented here is to identify prognostic markers and possible targets for immunotherapy.

Quantifying numbers of tumor infiltrating lymphocytes (TIL) can be viewed as a proxy measure for the intensity of an anti-tumor immune response *in vivo*. This approach has been used in many studies in large series of all major cancer types, gaining insight not only in the composition of lymphocyte infiltrates, but also in their associations with clinicopathological characteristics and patient survival. In chapter 2 we performed a systematic review, compiling all larger studies in which the prognostic significance of numbers of intratumoral CD3+, CD4+, CD8+, and FoxP3+ T lymphocytes was reported in solid malignancies. We performed meta-analysis to generate pooled estimates of survival outcomes for these T lymphocyte subsets. Importantly, these studies tend to have methodological differences, for instance in sample size, follow-up time, and methods of quantifying TIL and cut-off points. These factors may very well influence statistical outcomes and biological conclusions based on these outcomes. Therefore, we also tried to establish whether differences in methodology between studies were associated with the predictive value of TIL for patient survival.

In chapter 3 we report our own results regarding the prognostic impact of TIL in ovarian cancer in a large cohort of patients with a lengthy follow up time. We analyzed the presence of CD8+ CTL, FoxP3+ Treg, and CD45R0+ memory T lymphocytes in primary ovarian cancer tissue and in omental metastases from 306 ovarian cancer patients. Cell numbers were correlated to clinicopathological characteristics such as stage, histology, and residual tumor after primary debulking surgery, as well as patient survival.
An intact antigen processing and presentation apparatus is a prerequisite for an adequate anti-tumor T lymphocyte response. In particular, tumor antigens need to be cleaved into peptide fragments, loaded onto a Major Histocompatibility Complex (MHC) molecule, and transported to the cell surface. This cell surface bound complex of MHC molecule and peptide can be recognized by antigen-specific T lymphocytes, which subsequently execute their designated function. Antigen processing and presentation components are frequently downregulated in various types of cancer, providing an immune escape mechanism by impeding T lymphocyte recognition of tumor cells. In chapter 4 we analyzed whether this is an issue in ovarian cancer. To this end, we performed immunohistochemical stainings for components of the multicatalytic constitutive proteasome (MB1), the IFN-γ inducible immunoproteasome (LMP7), endoplasmatic reticulum associated transporter proteins (TAP1 and TAP2), chaperone proteins which facilitate loading into the MHC class I molecule (ERp57, ERAP1), and finally MHC class I molecules (HLA-A, HLA-B/C) and associated β2 microglobulin (β2m). Results from these stainings were subsequently correlated to clinicopathological factors, lymphocyte infiltration, and survival.

Tumor cells frequently arm themselves against the immune system by expressing immunosuppressive molecules. Human leukocyte antigen (HLA)-E could be one of these. HLA-E is a nonclassical Major Histocompatibility Complex (MHC) molecule, characterized by its nonpolymorphic nature. HLA-E binds the CD94 receptor in conjunction with either the inhibitory NKG2A or the stimulatory NKG2C molecule. These receptors are usually expressed on natural killer (NK) cells, but also on some activated CD8+ T lymphocytes. Thus, the interaction between HLA-E and CD94/NKG2A is a potential threat to the efficacy of the anti-tumor immune response. In chapter 5 we set out to determine the expression of HLA-E and its receptors on ovarian and cervical cancer samples. Moreover, we analyzed associations between HLA-E expression and (the prognostic significance of) CD8+ T lymphocytes and NK cells.

Identifying which factors determine the composition and magnitude of immune infiltrates in cancer could pave the way for immunotherapeutic interventions. In this context, chemokines could be influential. Chemokines are small molecules capable of inducing cell migration along a chemical gradient. They play important roles in for instance inflammation and infections, but also in the tissue formation and embryonic development. In cancer, they are implicated in attracting lymphocytes to the tumor, but also in angiogenesis and metastasis. In chapter 6, we examine the chemokine CXCL16 and its receptor CXCR6 in ovarian cancer. CXCL16 is a unique chemokine, because it exists not only in a soluble form (sCXCL16), but also as a transmembrane protein. sCXCL16 is generated via cleavage of transmembrane CXCL16 by metalloproteinases. We stained ovarian cancer tissue specimens for both transmembrane CXCL16 and CXCR6, and analyzed matching serum samples for sCXCL16. We determined associations between CXCL16 and CXCR6 expression, clinicopathological factors, and numbers of tumor infiltrating lymphocytes. Finally, we performed in vitro studies to assess the pathways involved in CXCL16 cleavage.
Another potential immunomodulatory protein is Galectin-9 (Gal-9). Gal-9 is a member of the galectin family of glycan binding protein, which is involved in embryonic development, tumor biology, and immune regulation. We found Gal-9 to be upregulated in ascites from ovarian cancer patients. Previous studies indicate that Gal-9 inhibits Th1 mediated immunity. However, in chapter 7, we report a novel immune stimulatory effect of Gal-9 on T lymphocytes. We performed various cell culturing experiments in which peripheral blood mononuclear cells (PBMCs) were incubated with low dosages of Gal-9 for seven days, which is considerably longer than previously reported. Next, we characterized changes in lymphocyte subsets in detail using flow cytometry.

Finally, in chapter 8, the results from this thesis are summarized and discussed, followed by an overview of future perspectives.

References

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