ENGLISH SUMMARY

Cervical Cancer

Cervical cancer is preceded by pre-malignant dysplastic changes in the epithelium known as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL). Affected cells comprising of more than one-third of the epithelium are called CIN 2/ 3 or high-grade SIL (HSIL). HSIL is caused by persistent infection with the human papillomavirus (HPV), a DNA virus infecting the basal cells of the cervical epithelium. There are over 100 types of HPV, which can be divided into low-risk (non-oncogenic) and high-risk (oncogenic) HPV. HPV is the most common sexually transmitted virus with a lifetime risk of infection of 80%. In the majority of cases infection is controlled after approximately 2 years, but persistence of infection occurs in about 10% of the women. These women are at risk of developing CIN, but other areas can be infected as well (vagina, anus, vulva, penis and/or head and neck) with risk of progression to invasive squamous(adeno)carcinoma. The HPV genome importantly encodes the two oncoproteins, the early antigens 6 (E6) and 7 (E7), which are expressed in HSIL and tumour cells which are required for the onset and maintenance of the malignant transformation.

Immunology

Keratinocytes are the first line of defense against invading pathogens. They express among other pathogen receptors, Toll-like receptor (TLR)-9, which can recognize HPV DNA. Activation of TLR9 should lead to activation of NF-kB, which results in the up regulation of proinflammatory cytokines (e.g. GM-CSF, IL-1b, TNF-a, IL-10, Il-12) and chemokine's, inducing the migration and the activation of antigen-presenting cells (APC's) e.g. Langerhans cells (LC's), Dendritic cells (DC's) and macrophages. Cues in the microenvironment will evoke APC to differentiate and migrate to the local lymphoid organs in order to present antigens to locally present naive T cells. Depending on different co-stimulatory or -inhibitory molecules and cytokine production (e.g., IL-12 or IL-10), a T-cell response will be induced which may comprise various CD8+ cytotoxic T cells (CTLs), CD4+ helper T cells (Th cells) and/or CD25+FoxP3+ regulatory T cells (Tregs). As HPV proteins are foreign to the body they should be able to trigger a strong immune reaction when presented in the cervical epithelium.

In the circulation of healthy individuals HPV-16 specific Th1, Th2 cells and CTLs are often detected against HPV. The importance of the adaptive immune system is shown by the high incidence of HPV infections, HSIL and cervical cancer in immune suppressed individuals. A broad HPV specific T-cell response, consisting of Th1, Th2 and CTLs, seems desired for viral control, yet in patients with HPV induced disease this response is often not present. It deems logical that restoration of this profile is one of the aims of immunotherapeutic strate-

gies. Failure of immune system to control infection is reflected by the fact that only one third of cervical cancer patients display a detectable systemic HPV-specific response against HPV, and when it is present, the response is generally not associated with the production of IFN-γ and consists of mostly Th2 cells, non-polarized T cells or Tregs. In the course of HPV infection to cervical cancer, various factors seem to play an important role. Pathogen recognition receptor (PPR) signaling in keratinocytes is suppressed, human leukocyte antigen (HLA) expression (important for the recognitions of cancer cells by lymphocytes) is down regulated, immunosuppressive cytokines are produced, Tregs are induced, while Th cells and CTLs may be rendered dysfunctional via the expression of co-inhibitory molecules. Furthermore, APC's are hampered in their function.

Immunotherapy of cancer

With all evidence pointing in the direction that failure of the immune system leads to the development of cervical cancer, restoration of an effective anti-tumour immunity seems the logical way forward. Various modalities have been developed with limited success, affecting often a minority of patients with progressive disease. As discussed above a multitude of mechanisms can be responsible for the tumours escape from the hosts' immune system. Therefore successful immunotherapy probably lies in multiple therapeutic strategies aiming at the enhancement of immune-mediated tumour destruction as well as simultaneously counteracting the tumour-induced immune suppression. Three main modalities have been developed to achieve this goal, therapeutic vaccination, antibody or cytokine therapy and adoptive cell transfer, but it is likely that this arsenal will increase tremendously in the coming years.

This Thesis

In the face of developing therapeutic vaccination strategies, it is of vital importance to gain a better understanding of the local tumour environment and the pre-existing local anti-tumour response. This thesis firstly investigates the natural immune response against HPV in patients with (pre-) cancerous lesions of the cervix with an emphasis on the tumour microenvironment. The last two chapters describe two clinical trials in which patients with pre-cancerous lesions of the cervix are vaccinated with an HPV16 E6/E7 synthetic overlapping long-peptide vaccine (HPV16-SLP).

In Chapter 2 we show that failure of the immune system is already present in a pre-cancerous phase. Only a minority of the patients with HPV16+ HSIL have an IFNγ associated HPV16 specific T cell response. Proliferative responses were found more often, especially in patients with persisting infections after previous surgical treatment. Moreover, we showed that these premalignant lesions could be infiltrated with HPV-specific Tregs. The results of this chap-

ter formed a good basis for the design and interpretation of immunotherapeutic vaccine approaches as treatment modality for HPV-induced in CIN.

In Chapter 3 we comprehensively analyzed the spontaneous tumour-specific immune response in patients with cervical cancer. We describe a large repertoire of HPV-specific T cells present in the tumour and lymph nodes of cervical cancer patients, whereby we distinguished four different cytokine signatures. This work shows lymphocytes can be isolated and cultured from tumour tissue for use in adoptive cell transfer.

A tumour is surrounded by stroma which is made up of, among others, fibroblasts, blood and lymph vessels and various immune cells. The tumour cells, immune cells and the cytokines they produce form a precarious balance, which is called the tumour microenvironment. The focus of Chapter 4 was to further improve our knowledge of the local microenvironment. We show an important role for myeloid cells, whereby, a strong intraepithelial infiltration with CD14+CD33-CD163- myeloid cells is associated with a large influx of intraepithelial T lymphocytes and an improved disease-specific survival. Furthermore we identified various immune fingerprints by analyzing 40 different immune parameters in the tumour microenvironment which are associated with the survival of patients. Chapter 5 further investigates the influence of human cervical cancer cells on myeloid cell (monocyte) differentiation. It shows that cancer cells can either hamper monocyte to DC differentiation, or skew their differentiation towards M2-like (immune suppressive) macrophages. Blocking studies revealed that M2-differentiation is caused by tumour-produced PgE2 and IL-6. Furthermore, upon CD40 activation or interaction with Th1 cells, these tumour-induced M2-macrophages could be switched back to activated M1-like macrophages. Blocking of Il-6 or PGE2 (through COX inhibitors) together with vaccination aimed at the induction of HPV specific Th1 cells could be beneficial in changing the tumour microenvironment towards an pro-inflammatory tumour rejecting profile.

The last two chapters describe two clinical studies testing the HPV16 synthetic long peptide vaccine (SLP) in patients with CIN. Chapter 6 describes a placebo controlled randomized phase II study in patients with HPV16 positive high grade CIN (CINII/III). The aim of the study was to investigate the capacity of the HPV16-SLP vaccine to stimulate the HPV16-specific T-cell response and to enhance the infiltration of HPV16-specific type 1 T-cells into the lesions and HPV clearance. Vaccination of HSIL patients resulted in increased Th1 HPV16-specific T-cell immunity, yet encountered problems of accrual and unacceptable local side-effects. Chapter 7 investigates the capacity of a low dose of HPV16-SLP vaccine to induce an HPV16-specific T-cell response in patients with low grade abnormalities of the cervix, to determine the long term memory response after vaccination and to evaluate the need and potency of a booster vaccination after one year. We concluded that two low

dose injections of HPV16-SLP can induce a strong and stable HPV16-specific Th1 T-cell response that lasts at least for 1 year. The booster injection resulted in increased Th2 responses. In order to further develop the HPV16-SLP in this group of patients it is essential to reduce the side-effects.

The future of immunotherapy in cervical cancer

So what is the way forward for immunotherapy in cervical cancer? Our and other studies on the mechanisms underlying the generation of anti-tumour responses and the immune evasion by tumours have underscored that multiple mechanisms restrain the host's immune system to rise to the challenge of combating the tumour. On the other hand favorable immune profiles have been highlighted that need boosting in order to keep the balance in favor of tumour eradication. Therapy should combine various synergistic approaches, and old and new therapies should be used side by side in order to enhance vaccination efficacy and counteract tumour suppression. Continued research in pre-clinical and clinical settings within our group is investigating the possibilities of using HPV16 SLP vaccination in combination with other adjuvants such as IFNα and Imiquimod, or a TLR2 ligand conjugated to the HPV16-SLP. Furthermore intradermal injection or delivery by tattooing are being further investigated as a possibility of avoiding the use of Montanide. Preliminary data within our group show that Tocilizumab, can functionally block the IL-6 receptor in patients with ovarian cancer. IL-6 is produced in cervical cancer affecting myeloid cell populations, further research to its use in cervical cancer patients is warranted. Preliminary results within the group shows that the current standard therapy (carboplatin + paclitaxel) for cervical cancer, normalizes the myeloid cell population as well as synergizes with therapeutic vaccination. Alternatively, one could enhance the number of tumour-specific T cells via adoptive cell transfer of ex-vivo cultured tumour-infiltrating lymphocytes as we showed in chapter 3. Current studies show it is feasible and a phase 1 clinical trial is being discussed. Other possibilities lie in the blocking of inhibitory receptors. In cervical carcinoma PD-1 is brought to expression in about half of the infiltrating CD8 T cells, suggesting that the blocking of PD-1 or its ligand PD-1L could have therapeutic benefits. Agonistic antibodies to co-stimulatory receptors can also be considered. We showed that CD40 can stimulate a shift from M2 to M1 in the presence of IFN-γ. Combining a monoclonal antibody to CD40 with a vaccine or other standard treatments *e.g.* surgery, radiation or chemotherapy should be further investigated in cervical cancer.

Our ongoing research has led to new insights into the role of the immune system in HPV induced disease and to various immunotherapeutic options which are being tested in pre-clinical and clinical trials. A future of possibilities lies ahead, all new immunotherapeutic strategies and combinations of therapies need extensive and accurate exploration as to dose optimization, interaction, timing of delivery and feasibility.