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Discussion

Future perspectives of
immunotherapy in cervical cancer



Conventional therapies like surgery, radiotherapy and chemotherapy, are of essential clinical value in the combat against cervical cancer. Yet, recurrent and metastatic disease are still often incurable due to limitations of toxicity and loss of efficacy of treatment. Based on new insights, novel immune-based therapies are gaining a undisputed place in the treatment of many cancers. Understanding the mechanisms underlying the generation of tumour immunity is vital if immunotherapeutic strategies are to join ranks in standard cancer therapy. This thesis contributed to the ongoing research into the role of the immune system during the development of cervical cancer and the search for effective and innovative immunotherapeutic strategies to combat it.

Previous studies in healthy individuals show that systemic HPV16 specific T-cell responses are frequently present in the healthy population (+-80%) (1, 2) indicating that a successful defence against HPV16 infection is commonly associated with the presence of a systemic effector T-cell response against these viral antigens. Our studies revealed that the HPV-specific T cell response is different at multiple levels in patients with HPV induced disease (systemically and locally), varying from no response, or a dysfunctional response, to an anti-inflammatory response in the presence of Tregs. In patients with cervical cancer, HPV16 specific systemic immune responses are detected in only about half of all patients (3) and are predominantly not associated with the production of pro-inflammatory cytokines. We found that this immune failure against HPV starts earlier and is already present at the time of pre-cancerous disease (HSIL) (chapter 2). We found an HPV 16 specific proliferative T-cell response against HPV16 in a minority of patients, and similar to cervical cancer patients, these T-cell responses mostly lacked a pro-inflammatory signature.

Tumours can mediate systemic and local effects in their effort to escape the immune system. Circulating HPV-specific lymphocytes have to home to the lesion and overcome the often hostile anti-inflammatory tumour microenvironment. Previous *in situ* immunohistochemical studies suggest that CD8+ T cells fail to migrate into the tumour cell nests and when they did this it usually coincided with the infiltration of regulatory CD4+ T cells (4). Yet immunohistochemistry cannot define the tumour specificity of these infiltrating lymphocytes. TIL cultures suggest that only 50-60% of the HPV16 or 18-positive cervical carcinoma, and 4 out of 7 HSIL, contain no HPV specific T cells in the tumour or its draining lymph nodes ((5) chapter 2). When HPV specific T cells were found they comprised not only HPV-specific effector cells but also HPV-specific regulatory T cells ((6); chapter 2). In the face of developing therapeutic vaccination strategies, it is important to gain a better and more complete understanding of the local tumour environment and the preexisting local anti-tumour response. This led to our in depth analysis of the spontaneous local tumour-specific immune response whereby we dissected the HPV E6- and E7-specific CD4+ and CD8+ T-cell responses down to the level of the percentage, specificity, cytokine polarization and number of different responding T-cells (chapter 3). We discovered that large polyclonal

repertoires of HPV-specific T cells can be present in tumours. At that time, we called these T-cells poised and non-functional yet in retrospect many tumours do contain HPV-specific T cells that seem well polarized and are able to produce IFN γ and IL-2 when stimulated *ex vivo* with their cognate antigen. This again shows the diversity of local HPV-specific responses varying from no HPV-specific T-cells, to regulatory T-cells, to dysfunctional or poised T-cells. Overcoming this immune failure is the key to successful immunotherapy and we found that the effector function of infiltrating T-cells could be enhanced when cultured in the presence of TLR ligands, such as PAM3CSK4 or poly(I:C) (chapter 3). The use of immune activating compounds, like TLR ligands, could have beneficial effects for therapeutic strategies. Imiquimod, which is used in the clinic for the treatment of genital warts and VIN, is an immune response modifier which triggers the TLR7 receptor. In patients with VIN the majority that responded to treatment had a pre-existing systemic HPV specific T-cells response (7). Although the local HPV specific T cell response was not investigated it seems plausible that this pre-existing HPV-specific T-cell response was activated in response to treatment.

It is clear that the tumour microenvironment is a precarious balance between tumour cells, infiltrating immune cells and the cytokines they produce. One of the hallmarks of cancer is the influx of myeloid cells which can be found infiltrating tumours in great numbers. In chapter 4 we aimed to improve our current understanding of the local microenvironment in cervical carcinomas. We focused on the constitution of tumour-infiltrating myeloid cells (TIM) and their relationship to other tumour-infiltrating immune cells, tumour characteristics and the disease-specific survival of patients with cervical cancer. Extensive literature demonstrates that high numbers of TAM facilitate tumour growth, disease progression and poor prognosis in various cancer types as reviewed by Heusinkveld et al (8). However, in CxCa, TAMs were never associated with clinical parameters or clinical outcome (9-11). Quantification of myeloid cell populations revealed a large variety between patients in type and amount of TIM, varying from no myeloid cells, to an abundance of mature or immature M1 and M2 macrophages. Analysis showed that a strong intraepithelial infiltration of matured M1 macrophages (CD14+CD33-CD163-), is associated with significantly improved disease-specific survival and is an independent prognostic factor as determined by multivariate analysis. Moreover, analysis with the CTL/Foxp3 ratio revealed a substantial increase in survival in the group of patients with tumours displaying dense intraepithelial matured M1 macrophage infiltrate and a high CD8+/Foxp3+ T-cell ratio. This work shows the importance of taking the whole tumour microenvironment into account and offers a profound insight on the important role of myeloid cells in the microenvironment and how they can work side by side with T cells to control tumours. Extensive studies in colorectal cancer have accentuated the importance of the number, function and location of infiltrating immune cells in the tumour microenvironment, leading to the development an immune score with a strong correlation to survival (12-14). Indeed in colorectal tumors B and T

cell infiltration can be linked to the production of IL-15, which in turn can be traced back to chromosomal changes in the tumor, underling the fact that multiple mechanisms can be involved in the creation of the tumour microenvironment. We succeeded in finding immunological fingerprints for cervical cancer by performing unsupervised clustering using 40 different immune parameters of the tumour microenvironment. The main determinants for a better survival were the presence of matured M1 macrophages and a high CD8+/Foxp3+ T-cell ratio, both independent prognostic factors. We found that the tumour-infiltrating T-cells are less able to exert a proper antitumour effect within a tumour microenvironment that does not allow the accumulation of high numbers of M1 macrophages. Patients with a better survival often have tumours that are infiltrated with relatively high numbers of M1 macrophages and displayed a high CD8/Treg ratio.

In view of our findings, selective inhibition of M2 macrophages together with the stimulation of M1 macrophages would seem a possible beneficial therapy in cervical cancer. In order to explore this possibility we analysed the effect of tumour cells on myeloid cell differentiation in order to explore the possibilities of reprogramming the abundantly present M2 macrophages toward an M1 phenotype. Our *in vitro* analysis in chapter 5 shows that cancer cells can hamper DC differentiation and function and can induce M2-like macrophages by the production of IL-6 and PGE2. Blocking these two cytokines during the differentiation period of the monocyte prevented the differentiation of monocytes to M2 macrophages. Furthermore, fully polarized M2 macrophages could switch to M1 macrophages when interacting with Th1 cells. A combination therapy consisting of COX inhibition, IL-6 blocking, and the induction of a strong Th1 T cell response could be a promising form of immunotherapy for the treatment of CxCa. COX-inhibiting drugs and mAbs to IL-6 receptor are used in the clinic for treatment of autoimmune diseases and further exploration of their use in the treatment of cancer is underway in our group. HPV-specific Th1 T-cell responses can be elicited by therapeutic vaccination.

Vaccination is a powerful method to induce humoral and cellular adaptive immune responses. The treatment of cancer strongly depends on the activation of antigen-specific CD4 and CD8 T cells with the ultimate aim of destroying the tumour cells. The capacity of the immune system to combat cancer is shown by the approval of a cell-based vaccine for the treatment of prostate cancer (Provenge) and an aspecific T-cell stimulating therapeutic antibody for the treatment of melanoma (Ipilimumab). Synthetic peptide vaccines were initially developed in order to elicit tumour-specific CTL responses and consisted of short peptides. These early vaccine were able to elicit CTL responses able to protect against tumour challenge in preclinical studies, yet in time after an initial expansion phase could result in functional deletion of the antigen-specific T cells, leading to enhanced tumour outgrowth (15). The discovery that long peptides prevented tolerance induction, induced CD4 helper responses and increased the diversity of the anti-tumour response, which could help reduce selective tumour antigen-loss during treatment (16), led to the development of

an HPV16 overlapping long synthetic peptide vaccine consisting of the whole length of the oncogenic proteins E6 and E7 emulsified in Montanide (17). Montanide ISA-51 is a water in oil emulsion that is frequently used with peptide vaccines as adjuvants. It's main function is a depot formation which inhibits immediate systemic bio-distribution of the peptides (which can lead to the cytokine release syndrome) and improves uptake by APCs. The group previously reported that vaccination with HPV16-SLP (25–35 amino acids) was highly immunogenic in end-stage cervical cancer patients (18, 19) and could result in complete and durable regression of human papilloma virus-induced premalignant lesions of the vulva by induction of a strong and broad multifunctional CD4 and CD8 T-cell reaction (20, 21).

Our first placebo-controlled study was in a group of patients with HSIL (CIN3 trial). Vaccination in patients with precancerous lesions has a distinct advantage as the development of cancer can be prevented. The study was set up to evaluate the local response after vaccination, but we ran into motivational problems in this patient group for whom there is a treatment available. Furthermore the patients experienced considerable systemic (flu-like symptoms) and local side effects (redness, swelling, itching and pain) which caused drop-out leading to premature closure of the study. The side effects had been expected as they were seen in our previous trials, yet in this group they seemed more severe and were less well accepted in the light of the fact that patients with a HSIL experience no symptoms of their lesions and have an available therapy. However, this was the first placebo-controlled trial with the HPV16-SLP vaccine and although the numbers were small, it allowed us to show that vaccination compared to the standard care, which includes a biopsy, can induce a broad and strong HPV16-specific response associated with the production of IFN γ , as measured by ex vivo IFN γ -ELISPOT and it showed that the responses are detectable by a skin test.

Our second randomized controlled trial included patients with low grade lesions of the cervix (CIN1 trial). The purpose of this trial was to study the long term memory response to the vaccine. In addition, as in this trial a lower vaccine dose was used it also allowed us to evaluate if this would lead to less side-effects without loss of immunogenicity. The differences in the HPV16-specific T-cell responses detected between the patients in the vaccine and placebo groups clearly showed that the lower dose HPV16-SLP vaccine is responsible for a strong HPV16-specific T-cell response after vaccination. This response, still detectable after one year, was boosted by re-vaccination. However a rise in Th2 type cytokines were seen, possibly warranting the addition of a polarizing adjuvants. Though less severe than in the HSIL group, the side-effects found in some patients were still difficult to accept. Especially the delayed local reactions at the vaccination sites, which included sterile abscesses, occurring several weeks to months after vaccination are cause for concern. Montanide ISA 51 is not a component of any approved human vaccine, but has been used in many previous trials. Recently more reports have been published outing concern about severe injection-site reactions with occasional sterile abscess formation (22-24) These side-effects

may be acceptable in the treatment of patients with (recurrent) cancer, yet to patients with pre-clinical lesions they are unacceptable. Based on these data, new clinical trials have been set up to test the immunogenicity of the HPV16-SLP when injected intradermally without montanide, and a trial in which the vaccine peptides are conjugated to a TLR2 ligand and injected intradermally.

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 favourable immune profiles have been highlighted that need boosting in order to keep the balance in favour 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. In order to boost the T cell response to CxCa one should put further effort in the combination with better adjuvants, delivered separately or conjugated to a vaccine. 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 IFN α (25) and Imiquimod (TLR 7), or a TLR2 ligand conjugated to the HPV16-SLP (unpublished data). Based on a study where healthy individuals showed an HPV 16 specific T-cell response after placement of a skin test, intradermal injection or delivery by tattooage are being further investigated as a possibility of avoiding the use of Montanide. Preliminary data within our group show that tocilizumab, used in the treatment of rheumatoid arthritis, can functionally block the IL-6 receptor in patients with ovarian cancer. As cervical carcinoma, similar to ovarian cancer, produces IL-6 which is linked to a worse survival (26, 27) further research to its use in cervical cancer patients is warranted. Current studies within our group show alterations in the number and phenotype of circulating and local myeloid cells in both an animal model for CxCa and in patients with CxCa when compared to controls. The current standard therapy (carboplatin + paclitaxel) normalizes the myeloid cell population as well as synergizes with therapeutic vaccination (Welters & van der Sluis & van Meir, in preparation) 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 (28, 29), 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 are being investigated in clinical trials in patients with melanoma, haematological malignancies, pancreatic and prostate cancer as

reviewed by Khong et al (30) and VonderHeide et al (31) and 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 optimisation, interaction, timing of delivery and feasibility. We should proceed with optimism, yet with great care.

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