Immunology and Immunotherapy of high grade cervical lesions and cancer

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Chapter 1

General Introduction

Immunology and Immunotherapy of Cervical cancer

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 (1-3). There are over 100 types of HPV, which can be divided into low-risk (non-oncogenic) and high-risk (oncogenic) HPV (4). HPV is the most common sexually transmitted virus (5) with a lifetime risk of infection of 80% (6-8). In the majority of cases infection is controlled after approximately 2 years, but persistence of infection occurs in about 10% of the women (6- 7). 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 (5-7, 9, 10). 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 (4).

IMMUNOLOGY

The immune systems defense against HPV-induced cervical lesions.

Both the innate and adaptive immune system play an important role in the protection against HPV. Keratinocytes, the first line of defense 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 (eg 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. Activation of the adaptive immune system requires viral antigen to be cross-presented by activated APC's (11). 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) (11). 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 detected against a broad array of epitopes of the viral early (E2, E6, E7) and late (L1) antigens.

These cells are able to leave the circulation and migrate to areas where the antigen is present (12-19). The importance of the adaptive immune system is shown by the high incidence of HPV infections, HSIL and cervical cancer in immune suppressed individuals (20) and at the time of spontaneous regression of HPV-infected genital warts, lesions are infiltrated with CTL, CD4+ T cells and macrophages (21). 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 strategies.

Escape from the immune system

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 E6 and E7, 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 (14, 22-26). 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 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.

- Viral recognition receptors

Keratinocytes are the first line of defense against invading pathogens. Viruses are recognized via several PRR. The viral DNA of HPV can be recognized by cells expressing TLR9. TLR9 is expressed in differentiating keratinocytes, but not in the undifferentiated cells of the basal layer which is the port d'entree of HPV. During infection with high risk HPV, TLR9 expression in the epithelium is not affected (2, 27), but its downstream signaling, as well as that of several other PRR which might recognize parts of HPV, is suppressed by the upregulation of the cellular protein ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) that dampens the production of interferon's, cytokines and chemokine's needed for the attraction and full blown activation of the adaptive immune system (2, 28). This renders the virus less visible, allowing HPV to persist.

- HLA expression

Essential elements for successful recognition of infected (or transformed) cells by the immune system is the presentation of viral peptides by HLA class I and II molecules. Down-regulation of HLA class I on HPV transformed cells, possibly resulting in escape from CTL attack, is observed in cervical neoplasia (29, 30) and in patients with cervical cancer where the loss of HLA-A expression is associated with worse survival (31). HLA class II expression is found on most tumour cells, but it is not clear whether this leads to recognition by Th

cells, which are needed for the promotion of CTL recruitment and cytolytic function (32). In colorectal carcinoma HLA class II expression is a favorable prognostic marker (33) but its role in cervical cancer is less clear. Other non-classical HLA types have been described to play a role in the persistence of HPV induced lesions. HLA-G is found on cervical tumour cells (34-35) and might inhibit the function of NK cells (not frequently found in HPV induced lesions) and T cells through the binding of inhibitory receptors. The expression of HLA-G is associated with progression of pre-malignant to malignant lesions (34, 36). HLA-E is another non-classical HLA type which is over expressed in HPV induced lesions. HLA-E can inhibit the function CTL's expressing the CD94/NKG2A receptor which are frequently found in HPV induced lesions (37). A third non-classical HLA molecule is the MHC class I chain related molecule A (MICA) which interacts with the stimulating NKG2D receptor on CTL's and NK cells and enhances the effector function of these cell types (31, 38). MICA is expressed on normal epithelium but is weak or absent in about 60% of cervical tumours and is associated with worse survival when analysed in combination with a low ratio of CTL/ Tregs (31).

- Tumour expressed inhibitory molecules

T cell infiltration and function can be hindered through tumour expressed molecules. Versican is expressed in cervical tumours and thought to prevent the homing of T cells into the tumour. High expression was associated with low numbers of tumour infiltrating T cells (TIL's) in cervical tumours (39). Furthermore, co-inhibitory molecules expressed by chronically activated CD4+ and CD8+ T cells can interact with their ligands, when locally expressed. Examples are Cytotoxic T-Lymphocyte Antigen 4 CTLA-4, program death 1 (PD-1) and T cell immunoglobulin mucin-3 (TIM-3) expressed on T cells (40-42). The interaction between PD-1 receptor on T cells and program death ligand 1 (PD-L1[B7-H1]) and/or PD-L2 (B7-DC) results in the induction of apoptosis, anergy or exhaustion of effector T cells (43-45). Many PD-1 positive T cells are found infiltrating cervical cancer, yet its ligand PD-L1 is found less frequently on the cancer cells (45, 46). Interaction of TIM-3 and its ligand Galectin-9 results in a decreased Th1 and CTL immunity by inducing apoptosis of Th1 cells as well as by inhibiting the function of CTLs and Th1 cells (40, 42, 47). Increase in Galectin-9, has been associated with malignant differentiation in cervical cancer (48).

- Antigen presenting cells

HPV has been reported to also regulate the function and migration of APCs present in the epithelia, thereby hampering the activation of the adaptive immune response. The L2 protein of HPV is able to suppress the maturation of LC's causing inadequate antigen presentation to T cells (3, 49). Not only the function, but also the number of LCs seems reduced in HPV-infected epithelia (50-52). Furthermore, the absence of pro inflammatory signals in HPV-infected epithelia can result in inappropriately activated APCs (53). The

expression of HLA class II on tolerogenic APC's in the tumour could have pro-tumoural effects by rendering Th cells anergic through the lack of stimulation via co stimulatory molecules (eg CD40) and/or by the production of inhibitory cytokines, or by the induction and activation of Tregs (54). Tumour associated macrophages (TAM), are a heterogeneous myeloid cell population originating from monocytes in the circulation, which are able to undergo specific differentiation depending on the different stimulatory signals within the tumour microenvironment. Macrophages display the flexibility to continuously adapt to such cues in the local microenvironment allowing them to differentiate into cells that phenotypically and functionally fit between the two extreme polarization states known: M1-like pro-inflammatory, tumoricidal macrophages (M1) and M2-like anti-inflammatory, tumor-promoting macrophages (M2). Both are frequently found infiltrating tumours, but opposed to M1, M2 have a poor antigen-presenting capacity, preventing T-cells to be properly activated, furthermore they can enhance angiogenesis and metastasis (55) and have been associated with worse survival in various tumour types (56).

- Regulatory T cells and cytokines

In addition to CTL and Th cells the tumour milieu may also comprise Treg. Tregs display a high expression of CD25 and express the forkhead box protein 3 (FOXp3). Tumour infiltrating FOXp3+ T cells have been studied extensively and have been correlated with a poorer prognosis in various cancer types (57-64). Tregs found in tumours can inhibit the proliferation and function of Th cells and CTL's by preventing the expression of Il-2 receptor or inhibiting Il-2 production (24). The ratio of tumour-infiltrating CD4+, CD8+ and tumour-infiltrating Tregs is strongly correlated to survival in patients with cervical cancer (24, 31, 65, 66) and HPV specific Tregs have been found infiltrating cervical cancer (24, 25). Tregs further suppress the antitumor response by the induction of suppressive M2 macrophages, the up regulation of Il-10, the induction of tolerogenic indoleamine 2,3-dioxygenase (IDO)-positive APCs and the production of TGFβ (24). Il-10 and TGFβ can also be produced by tumour cells. Together, tumour cells, TAM and IDO+ APC may form a strongly immunosuppressive milieu. IDO, which is an immunosuppressive molecule, was found to be highly expressed in HSIL and cervical cancer on myeloid cells in the tumour stroma and the expression of IDO by tumour cells was correlated with an unfavourable survival in patients with cervical cancer (67-69). TGFβ is highly expressed in SIL and cervical cancer (70, 71), and prevents the infiltration of T cells into tumours, inhibiting their activation and inducing Tregs (72).

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, but it is likely that this arsenal will increase tremendously in the coming years.

Therapeutic vaccination

Various therapeutic vaccines aiming at inducing and/or restoring the tumour-specific T-cell response have been investigated (73). Vaccines based on recombinant viral vectors, recombinant proteins, DNA, antigen-pulsed DC's and peptides have been developed. For cervical cancer two promising vaccination strategies have been developed. One is a recombinant HPV16 E6E7L2 fusion protein (tissue antigen-cervical intraepithelial neoplasia, TA-CIN) (74) showing clinical responses when the vaccine was combined with Imiquimod treatment of the lesion. The other is a synthetic long overlapping peptide vaccine of HPV16 E6 and E7 (HPV16 SLP) which showed a good immunogenicity in cervical cancer patients (75, 76) and a clinical response in patients with VIN (77). This clinical response was associated with the induction of a strong HPV-specific CD4+ T cell and CD8+ T cell response. Furthermore, unresponsive patients showed an increase in the numbers of HPV16-specific Tregs infiltrating the lesion.

Antibodies and cytokines

Nonspecific immune stimulation with the use of monoclonal antibodies or recombinant cytokines can activate the immune system directly or counteract tumour induced immune suppression. Blocking or inhibiting immune inhibitory pathways are widely being investigated in cancer immunotherapy. CTLA4 blockade with Ipilimumab results in a broad enhancement of the immune system by T-effector stimulation and depletion of Tregs as reviewed by Blank et al (78). Blocking of CTLA-4 has been found to mediate a significant mean survival benefit in patients with advanced melanoma and an impressive long-term survival rate of a subgroup of patients (18% surviving longer than 2 years) (79). PD-1 is another immune checkpoint molecule, expressed by activated effector or Tregs within tumours (45, 80, 81), while its ligand, PD-L1, is expressed by tumour cells of many different tumour types and on macrophages and DC's (44, 45). Interaction results in the inhibition

of T-cell function. Blockade of this pathway (Nivolumab) has been demonstrated to have success in pre-clinical studies (82). Phase 1 trials are showing promising response rates with Anti-PD-L1 and Anti-PD-1 antibodies with less toxicity that CTLA-4 (83, 84). Combining Ipilimumab and Nivolumab showed objective responses in more that 40-50% of melanoma patients (85). The stimulation of costimulatory receptors on the other hand is also a promising potential strategy. OX40 (CD134) and 4-1BB (CD137) are co stimulatory receptors of the TNF family that are expressed on activated CD4 and CD8 T-cells. Agonistic antibodies to these receptors have powerful capabilities to activate CD8+ T cells to produce IFN-γ (86, 87). Various recombinant cytokine have found their way in to clinical trials with promising results. Examples are GM-CSF, inducing the proliferation of DC's and macrophages, Il-2 inducing T-cell activation and proliferation (88), Il-12 creating IFNγ producing T-cells (89) and type I Interferons which are known to have direct anti-viral properties, induce the differentiation of Th cells, generate CTL's and prolong the survival of all T cells (90). Other potential candidate cytokines are Il-7, Il-15 and Il-21 able to stimulate T cells or chemokines (e.g. CXCL9 and CXCL10) to attract T cells to the tumour.

ADOPTIVE CELL THERAPY

In adoptive cell therapy (ACT), lymphocytes are ex-vivo isolated from peripheral blood, tumour-draining lymph nodes or the tumour of cancer patients. Following an in vitro expansion the (partially) tumour-specific lymphocyte population is re-infused into the patient. This strategy may offer advantages over in vivo vaccination as the T cells are activated and expanded outside the cancer-induced immunosuppressive milieu, potentially allowing them to acquire more potent anti-tumoural properties. Moreover, in vivo lymphocyte depletion prior to infusion may also deplete pre-existing Tregs. In melanoma patients ACT has led to significant and durable remissions as reviewed in Phan *et al* (91).

OUTLINE OF THIS THESIS

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, by studying the local HPV-specific T-cell responses in HSIL and cervical tumours and draining lymph nodes, the presence of myeloid cells in cervical tumours, their interaction with the microenvironment and the effect on survival. 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 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. In **Chapter 2** we investigated the systemic and local HPV16 T cell response in patients with HSIL. Only a minority of the patients with HPV16+ HSIL have an IFNγ associated HPV16 specific T cell response. Proliferative responses were more often found, 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 chapter formed a good basis for the design and interpretation of immunotherapeutic vaccine approaches as treatment modality for HPV-induced in pre-cancerous lesions.

Previous work showed that HPV specific T cells can be found infiltrating the tumour and its draining lymph nodes. We wanted to know the potential of these cells to aid tumour immunity and, therefore, studied the properties of these T cells. In **Chapter 3** we comprehensively analysed the spontaneous tumour-specific immune response in patients with cervical cancer by dissecting local 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. We describe a large polyclonal repertoire of HPV-specific T cells present in the tumour and lymph nodes of cervical cancer patients, whereby we distinguished four different cytokine signatures based on the production of IFNγ and Il-2. This work shows TIL's can be isolated and cultured for use in ACT.

The focus of **Chapter 4** was to further improve our knowledge of the local microenvironment by investigating the tumour infiltrating myeloid cells. The different types of myeloid cells, their clinical impact and their co-operation with T cells in the cervical cancer microenvironment was analysed. A strong intraepithelial infiltration with CD14+CD33-CD163- myeloid cells associated with a large influx of intraepithelial T lymphocytes, improved disease-specific survival and formed an independent prognostic factor for survival. This study provided a profound insight on the role of myeloid cells in the microenvironment, how they can work side by side with T cells to control tumours and forms a major addition to the current discussion about the impact of tumour-infiltrating myeloid cells in human cancers.

The influence of human cervical cancer cells on myeloid cell (monocyte) differentiation is presented in **Chapter 5**. It shows that the majority of cancer cells either hamper monocyte to DC differentiation, or skew their differentiation towards M2-like macrophages. Blocking studies revealed that M2-differentiation was caused by tumour-produced PgE2 and IL-6. Furthermore, upon cognate interaction with Th1 cells these tumour-induced M2-macrophages could be switched back to activated M1-like macrophages. These data show

the plasticity of tumour-induced tolerogenic APC and suggest that increased numbers of tumour-infiltrating Th1 cells may stimulate a tumour-rejecting environment by switching M2-macrophages to classical pro-inflammatory tumouricidal M1 macrophages. Vaccination aimed at the induction of HPV specific Th1 cells could be beneficial.

The aim of **Chapter 6** 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 of patients with HPV16+ HSIL and HPV clearance. This was a placebo controlled randomized phase II study in patients with HPV16 positive HSIL. Vaccination of HSIL patients resulted in increased Th1 HPV16-specific T-cell immunity.

Chapter 7 investigates the capacity of a low dose of the 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 finally 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 **Chapter 8** the contribution of our results to the current knowledge of the immune response to HPV induced lesions and cancer is discussed. In addition, the present and future of immunotherapeutic strategies in cervical cancer according to recent international literature is reviewed.

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