Regulation and subversion of HPV16-specific immunity in cancer patients

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Chapter 1

General introduction

Partially published in:
Human Immunology. 2008; 69(4-5):241-9

1. Introduction. Cancers arise as consequence of genomic mutations or infection with oncogenic viruses. The process of transformation from a healthy cell into a malignant tumor cell requires the acquisition of several characteristics, which are known as the six hallmarks of cancer (1). These hallmarks include self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis as well as tissue invasion and metastases (Figure 1).

Not only do these changes within the cell lead to uncontrolled growth, it also leads to altered protein-levels, to neo-expression of proteins or to the expression of foreign proteins in the case of oncogenic viruses. These proteins may therefore be potential targets of the immune response, leading to the eradication of the malignant cells. In order to evade recognition by the immune system from being destructed, tumors develop immune evasion mechanisms which have been recognized as the seventh hallmark of cancer (2, 3) (Figure 1).

During malignant transformation a close interaction exists between the tumor and the immune system, which can consist of three phases and is known as cancer immunoediting (4). During the first phase (the elimination or cancer immunosurveillance phase) the immune system is able to protect the host from a developing tumor. However, some transformed cells may escape the immunological pressure, thereby entering the second phase (the equilibrium phase). During this period of immune-mediated latency the tumor persists and acquires new mutations. This may allow the tumor to enter the third phase (tumor escape) during which established tumors become clinically manifest (reviewed in (5)).

2. Immunosurveillance of tumors

The role of the adaptive immune system in inhibiting tumor outgrowth has been well demonstrated in mouse models (6, 7). In these studies it was shown that mice deficient for functional T cells, spontaneously develop tumors. These spontaneous tumors are rejected by CD8 T cells when the tumors were transplanted to immunocompetent animals. However, in a model for spontaneous tumors non-functional CD8 T cells were induced (8), indicating that tolerance is induced already in early stages. In cancer patients the role of T cells in tumor surveillance has been illustrated by several studies showing that increased CD8 T-cell infiltration is associated with a better prognosis in a number of cancers (9-13). Moreover, immunocompromised individuals are at higher risk to develop tumors, including cervical cancer (14).

On the other hand, Natural Killer (NK) cells seem to play only a limited role in the immune surveillance of the primary tumor in cervical cancer patients, as only low numbers of CD57+CD3- cells, encompassing a subpopulation of NK cells, are infiltrating tumor tissue (10, 15). In concordance with these findings NKp46+ cells, encompassing all NK-cells, are rarely detected (Jordanova, unpublished data). Despite their absence at the tumor site they are present in vast numbers in the peripheral blood and in the lymph system, where they may kill metastasizing cells.

The T cells involved in immunosurveillance recognize specific antigens that discriminate tumor cells from healthy tissues. These antigens can either be tumor specific or tumor associated (16). Tumor-specific antigens (TSA) are either mutated self-antigens or antigens from viral origin. Tumor-associated antigens (TAA) can be either antigens normally expressed only in embryonic tissue, differentiation antigens which have restricted expression in specific tissues or antigens which are expressed in healthy tissue at low levels and in tumor tissue at higher levels. These antigens are potential targets for cancer immunotherapy.

TAA are expressed differentially in healthy tissues and can therefore be potentially recognized by CD4 and CD8 T cells. During the last decades over 100 immunogenic TAA have been identified (17) (www.cta.lncc.br; www.cancerimmunity.org). Examples of such antigens are Melan-A, gp100, SURVIVIN and NY-ESO 1. Most of these antigens are specific for a limited number of tumors. However, more universal immunogenic tumor antigens have also been identified, which include underglycosylated MUC-1, overexpressed hTERT and p53 (18-20).

Tumors harbor high numbers of genetic mutations, approximately 90 mutations are present within a tumor. Around 11 of these mutations are predominantly present throughout the tumor (21). These mutations result in the expression of altered proteins which contain potentially immunogenic tumor-specific T-cell epitopes. Frequently the mutations are essential in tumor genesis, as a result these mutated proteins are expressed in every tumor cell. Despite the abundant mutations in essential

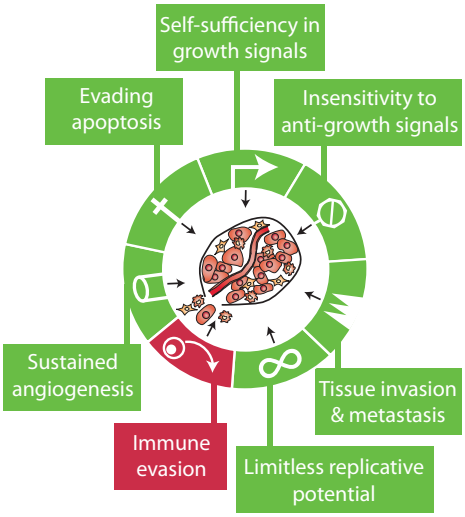

Figure1. Seven hallmarks of cancer. Modified from Hanahan and Weinberg (1).

proteins, relatively little is known about their mutation frequency and immunogenicity (22). The most well known genetic changes in malignant tumor cells are in p53. Mutations within the gene are frequently located in conserved residues, indicating altered biological function and potential good targets for immunotherapy (23). However, mutations in p53 are diverse within the gene and frequently represent unique mutations, making it difficult to design immunotherapy against mutated p53 (20).

Viruses are estimated to be involved in 15 to 20% of all human cancers (24). The accepted human tumorviruses encompass both DNA viruses (human papillomavirus (HPV), Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus and hepatitis B virus) and RNA viruses (hepatitis C virus and human T-cell leukemia virus) (24, 25). HPV and other tumor viruses persist in the cancer cells either by integration in the host genome or by remaining episomally (26). Since the viral oncogenes are essential in maintaining the malignant phenotype of the tumor cell, the viral gene products are expressed in every tumor cell. This wide expression pattern makes the viral proteins potential good targets for immunotherapy of cancer.

3. Cervical cancer and Human Papilloma Viruses

Cervical cancer is caused by HPV in virtually all cases and is the second most common cancer in women worldwide (27-29). The most prevalent type is high-risk type HPV16, which accounts worldwide for over 50% of the cases of cervical cancer. The second most prevalent type in the Caucasian population is HPV18, which accounts for more than 15%. Other high-risk types of HPV, of which over 15 have been identified, contribute substantially to cervical cancer as well (30).

HPV is a small double stranded DNA virus (7-8 kb), which can infect the basal layers of the epidermis and mucosal epithelium. The viral life cycle is tightly regulated to the cycle of the host cell. In the basal layers the proliferation-inducing early genes (including E6 and E7) are expressed, resulting in lateral expansion of the infected cells. After entry into the suprabasal layers the viral genes responsible for viral replication, structural proteins and viral assembly are expressed. Subsequently, infectious particles are released (Reviewed in (31, 32)) (Figure 2).

The properties of both E6 and E7 are essential for HPV-induced malignant transformation and are therefore known as viral oncogenes (33). Both proteins interact with multiple host proteins to promote cell proliferation and inhibition of apoptosis. E6 is well known for its ability to promote p53 and BAK degradation, thereby inhibiting apoptosis (34, 35). Additionally, E6 can also promote the activation of telomerase (36). E7 on the other hand is able to interact with the retinoblastoma family members and thereby it enhances cell proliferation (37). Moreover, E7 stimulates cyclin A and E as well, promoting G0/G1 progression (38).

The progression of HPV infection to cervical cancer is a slow process and can be divided in 5 stages (Figure 2). The first stage comprises of infection with HPV, in most infected individuals the virus is cleared within 2 years. However, in approximately 10% of the infections the virus persists (entering the second stage). The virus can persist for several years and is strongly linked to a higher risk for the diagnosis of low-grade squamous intraepithelial lesion (LSIL) (the third stage). This stage is characterized by mild dysplasia due to progression of persistently infected cells to precancer. This lesion can further progress into high-grade squamous intraepithelial lesion (HSIL), which is characterized by moderate dysplasia to in situ carcinoma (fourth stage). The HSIL can progress further into the last stage, invasive carcinoma (reviewed in (39)). During the first 3 stages, spontaneous regression and/or clearance is common. It has been estimated that less than 1% of the infected women develop cervical cancer (40, 41). Little is known on the progression versus spontaneous regression rates in HSIL since surgical intervention therapies are used to treat HSIL. However, the general acceptance is that HSIL do not regress spontaneously (42). Additionally, early studies

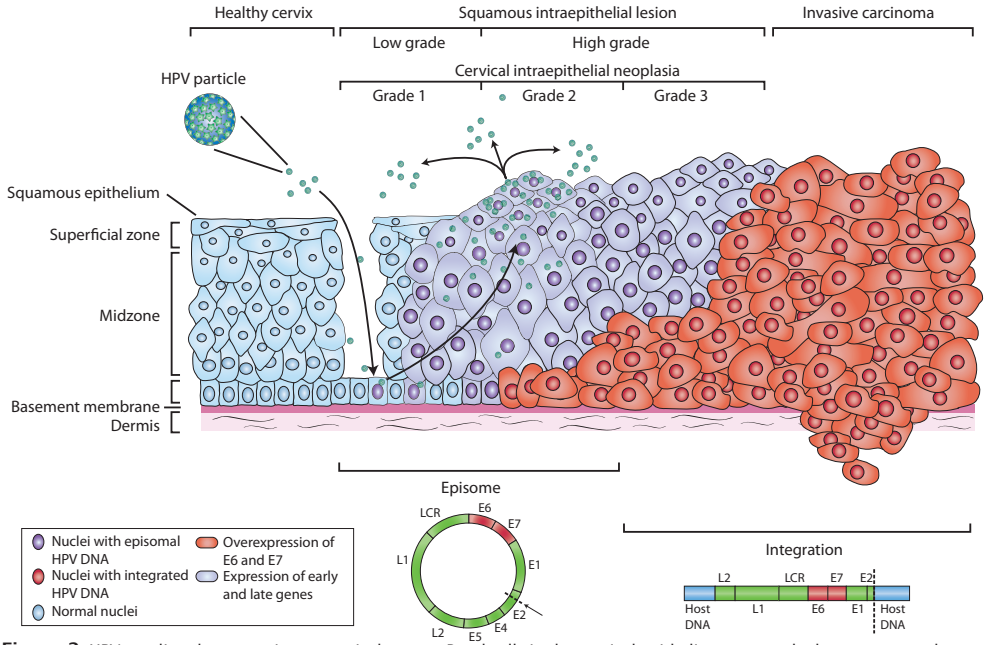

Figure 2. HPV–mediated progression to cervical cancer. Basal cells in the cervical epithelium rest on the basement membrane, which is supported by the dermis. Human papillomavirus (HPV) is thought to access the basal cells through micro–abrasions in the cervical epithelium. Following infection, the early HPV genes E1, E2, E4, E5, E6 and E7 are expressed and the viral DNA replicates from episomal DNA. In the upper layers of epithelium (the midzone and superficial zone) the viral genome is replicated further, and the late genes L1 and L2, and E4 are expressed. L1 and L2 encapsidate the viral genomes to form progeny virions in the nucleus. The shed virus can then initiate a new infection. Lowgrade intraepithelial lesions support productive viral replication. An unknown number of high–risk HPV infections progress to high–grade cervical intraepithelial neoplasia (HGCIN). The progression of untreated lesions to microinvasive and invasive cancer is associated with the integration of the HPV genome into the host chromosomes, with associated loss or disruption of E2, and subsequent upregulation of E6 and E7 oncogene expression. LCR, long control region. Modified from Woodman et al. (176).

suggested that less than 30% of HSIL progress further into invasive carcinoma within 10 years (43).

During malignant transformation, the DNA of HPV is able to integrate into the host genome at random positions (44, 45). The integration of the viral DNA is associated with the transition into invasive carcinoma (46). During insertion into the host DNA, the integrated DNA is either complete or there is partial loss of the viral genes. Loss of E1, E2, E4, E5 and L2 occurs frequently, increasing the immortalization potential of E6 and E7 (31, 47). Moreover, E6 and E7 are essential in maintaining the malignant phenotype of the tumor cells and are therefore expressed in every tumor cell (48). HPV E6 and E7 are therefore potential good targets for the immune system.

The role of the immune system in controlling HPV-infections is illustrated by the observation that strong proliferative HPV16 E2- and E6-specific T-cell memory responses are frequently detected in HPV-negative healthy women as witness of previous infection. These responses are accompanied by IFNg and IL-5 production and low levels of IL-10 (49-51). Similar findings have been described for HPV18 (52). T-helper responses against the C-terminal domain of HPV16E2 frequently occur at the time of virus clearance (53). Occasional responses against E7 are observed as well (50, 54).

In contrast, in cervical cancer patients HPV-specific T-cell responses are detected only in half of the CxCa and HSIL patients. In these patients a weak proliferative response was observed. This response was not associated with production of the proinflammatory cytokines IL-5 and IFNg, but the anti-inflammatory cytokine IL-10 was still detected in CxCa patients (51, 55). Similar results were found for HPV18 as well (52). Consistent with these results other studies report that HPV16-specific proliferative responses are occasionally observed whereas Th1 type responses, as defined by IL-2 production, are low or lacking in cervical cancer patients (56-59). In HSIL and CxCa patients HPV16-specific CTL can only rarely be detected in the peripheral blood (60-63), whereas such responses are frequently detected in healthy donors (64, 65). Since CD4 T cells are essential in the induction and maintenance of CD8 CTL immunity (66), the defective Th1 response in CxCa patients may explain the low levels of HPV-specific CTL. Furthermore, the CD4 T-cell response is accompanied by IL-10 production, indicating a role for active suppression.

4. Immune evasion strategies employed by tumors

During malignant transformation, a close interaction exists between the tumor cells and the immune system, which is described above as cancer immunoediting. This is a slow process that can take years or even decades. Because of a continuous immunological pressure on the tumor, the tumor develops several mechanisms to evade the immune system. In many tumors the transformed cells have acquired several mechanisms to directly protect them from immune cell mediated killing. These mechanisms include (A) anitgen loss, MHC class I downregulation and impaired antigen processing to prevent antigen presentation, (B) resistance to immune-mediated apoptosis, (C) the expression of immunosuppressive factors and (D) the attraction of immune cells that are able to inhibit the immune response (Figure 3). The different mechanisms described in the literature and their role in cervical cancer will be discussed below.

4.1. Direct evasion of the anti-tumor response

The occurrence of antigen loss has been well demonstrated in an immunogenic tumor mouse model (67, 68). These studies collectively show that tumor cells are able to lose the expression of antigens as result of immunological pressure. Occurrence of antigen loss has also been llustrated in melanoma patients. Antigens normally expressed by melanocytes are frequently lacking in tumor cell lines and tumor tissue from melanoma patients (reviewed in (69)). However, antigen loss is almost absent in cervical cancer patients, as HPV DNA can be detected in virtually all tumors and E6 and E7 RNA is present throughout malignant transformation in all cases (27, 70, 71). The E6 and E7 proteins are essential in maintaining the malignant phenotype of the tumor cells, which may explain the absence of antigen loss in HPV-induced cervical cancer.

The two major pathways used by lymphocytes to induce apoptosis in target cells are the granule exocytosis pathway and the FAS/FASL pathway (72). For these apoptotic pathways, tumor-escape variants have been described (73). Examples of such escape-mechanisms are overexpression of the anti-apoptotic gene BCL-2 (74), expression of the FASL-inhibitors FLICE-inhibitory protein (cFLIP) (75, 76) and expression of the Granzyme B inhibitor PI-9 (77). cFLIP has been shown to be overexpressed in cervical tumor tissue compared to healthy cervix, but the impact on survival is still unclear (78). Recently, SerpinA1 and SerpinA3 have been shown to be overexpressed in tumors of a subpopulation of cervical cancer patients. In this study overexpression of these proteins correlated with a poorer survival (79). Since SerpinA1 and SerpinA3 both have been implicated in inhibition of apoptosis (80, 81), overexpression of these proteins may render the tumor cells insensitive for immune mediated apoptosis.

Many tumors downregulate MHC-class I to evade recognition by the immune system. Downregulation of the HLA class I genes can originate from multiple mechanisms (Reviewed in (82)). Mutations of the individual HLA alleles together with the deletion of the common b2 microglobulin genes are commonly observed in many types of cancer. As another immune escape mechanism other tumors have defects in the antigen processing machinery. Defective antigen processing leads to impaired antigen presentation of tumor antigens, as a result TSA or TAA normally produced by the proteasome and transported through TAP cannot be presented on MHC class I (69). Defects in the antigen

machinery include decreased expression of proteasome subunits (eg. LMP2 and LMP7) and transporter subunits (TAP1 and TAP2). The frequencies of these defects differ between tumor types (83). Since antigen presentation of the HPV16E6 protein depends on TAP and the proteasome (84), defects in these proteins result in decreased recognition of tumor cells by HPV-specific T cells. E7 of the low-risk HPV11 has been implicated in TAP inhibition in laryngeal papillomatosis (85, 86), but this effect has not been reported for other HPV types. On the other hand, E7 of HPV6, -16 and -18 has been shown to reduce the expression of Class I heavy chain, LMP2 and/or TAP1 (87). HPV16 E5 has been shown to downregulate HLA-A and -B cell surface expression, but no decrease was found in total HLA class I expression (88). However, the exact mechanism by which HPV16 E5 modulates Class I surface expression is not known.

Despite direct interactions of HPV proteins with TAP, MHC class I is rarely completely lost in LSIL or HSIL lesions (89). Moreover, interference with TAP is detected in a subpopulation of the cervical cancer patients, indicating that the observed downregulation in the MHC class I pathway is not directly caused by HPV (90, 91). Interference of HPV proteins with MHC class I presentation machinery is therefore not likely to have a dominant role in cervical cancer patients. Alternatively, MHC class I defects may develop during malignant formation, due to immunological pressure on the tumor. In cervical cancer patients abnormalities in the MHC class I presentation machinery has been well documented (15, 89-97). Alterations in MHC-class I presentation pathway has been observed in approximately 90% of the patients (94). However, in this study total loss of MHC class I has been observed in only 10% of the patients, indicating that 90% of the cervical cancer patients could benefit from T-cell mediated immunotherapy.

4.2. Indirect evasion of T–cell mediated killing

Many tumors express inhibitory coreceptors (98). The inhibitory B7 family member B7-H1 (PD-L1) is expressed on a wide variety of tumors (99). This molecule can interact with PD-1 and CD80 on T cells, thereby inducing apoptosis, anergy or exhaustion of effector T cells (100). The PD-L1 expression has been studied in a number of cancer types and is generally associated with poor prognosis (reviewed in (100)). The recently identified B7-H3 and B7x have been found to be expressed on tumors and B7-H3 expression has been shown to be correlated to decreased survival in renal cell carcimoma (101, 102).

Immunosuppressive factors produced by tumor cells can also contribute to the immunosuppressive microenvironment. These factors include indoleamine 2,3-dioxygenase (IDO), VEGF, TGFβ and IL-10. The IDO pathway has also been implicated in indirect immune escape by tumors. The immune tolerant effect of IDO functions through the depletion of tryptophan and the generation of kynurenine metabolites, resulting in affected T-cell proliferation and survival (103). A few studies showed that IDO is expressed by the tumor and the level of expression is an independent prognostic factor in colorectal cancer (104, 105). IDO has been implicated to interfere with the initial immune response to tumor antigens, the cytolytic capacity of CTL and enhanced suppressive capacity of regulatory T cells (Tregs) (106). IDO has been shown to be present in HSIL and CxCa, but the functional consequence of IDO was not addressed (107). Therefore, the exact role of IDO in cervical cancer remains unclear. VEGF, which is normally involved in vessel formation, also contributes to the immune suppressive environment by the attraction of immature dendritc cells (DCs) and macrophages, which will be discussed below (108). TGFβ is expressed in many tumors and is known to inhibit immune responses at multiple levels (109, 110). In cervical tumors, TGFβ mRNA is frequently detected but does not correlate with survival (111). However, PAI-1 and αvβ6 integrin expression, which reflect the presence of active TGFβ, has a negative influence on survival (111, 112). Next to immune regulation, TGFβ also modulates other processes. These include cell invasion and metastatic colonization. The impact of TGFβ on immune escape is therefore difficult to determine in cancer patients (109). However, an inverse relation exists between TGFβ expression in tumors and tumor infiltrating lymphocytes, indicating that TGFβ may hamper the infiltration of lymphocytes in cervical cancer (113). HPV has also been implicated to induce the production of immunosuppressive factors. The E6 and E7 proteins have been reported to inhibit Interferon regulatory factor (IRF3 and IRF1 respectively), which are transcription factors involved in immune pathways (114, 115). Interference with these proteins results in an impaired IFN-pathway and thereby NFκB-stimulated genes. This results in lower levels of pro-inflammatory cytokines, which may be a direct mechanism by which HPV creates an immunosuppressive microenvironment (116).

4.3. Attraction of innate immune cells with immunosuppressive properties

A third mechanism of immune evasion by tumors is the attraction of immune cells with immunosuppressive properties. These include both members from the innate and the adaptive immune system that are able to suppress anti-tumor responses. Macrophages are recruited by tumors in high numbers, in these tumors they differentiate predominantly into a M2 phenotype (117, 118). These tumor-associated macrophages (TAMs) have direct effects on tumor growth, vascularization and modulation of the tumor stroma. Moreover, TAMs also produce a wide array of cytokines and chemokines, resulting in immune evasion at multiple levels. These evasive mechanisms include alteration of DC phenotype and modulation of T-cell responses (118). Tumor infiltrating CD68+ macrophages have been found to infiltrate cervical tumors and metastatic lymph nodes. The TAMs reach numbers equal to infiltrating T cells in tumors (95, 119, 120). However,

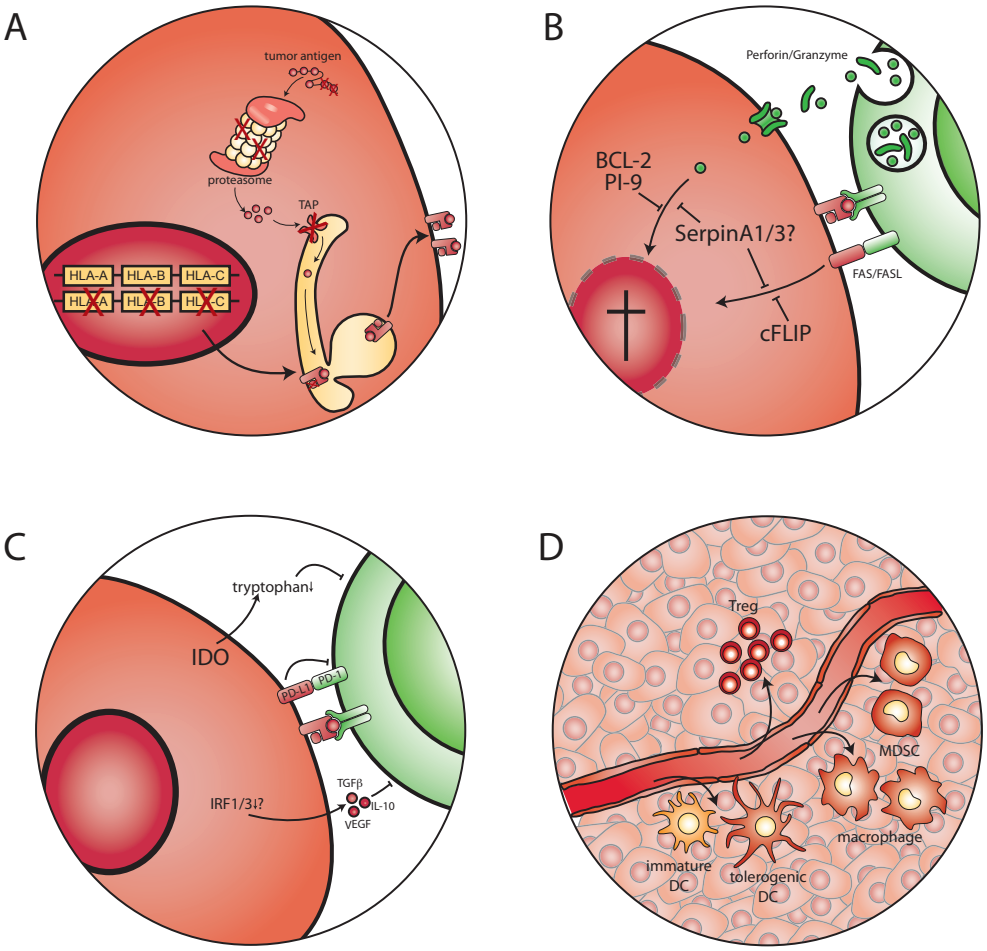

Figure 3. Immune evasion mechanisms employed by tumors. Many tumors the transformed cells have acquired several mechanisms to protect them from immune cell mediated killing. These mechanisms include (A) downregulation of the antigen presentation machinery, (B) insensitivity to CTL–mediated cytotoxicity, (C) production of immunosuppressive cytokines and (D) attraction of immune cells with immunosuppressive properties.

the type of macrophage and their impact on the immune system have not been addressed in these studies.

DCs are the key players in orchestration and initiation of the immune response. DCs have been shown to infiltrate human tumors. However, they usually have an immature phenotype as they lack costimulatory molecules (Reviewed in (121)). These improperly polarized DCs induce rather T-cell deletion and anergy as opposed to induction of effector T cells which are able to eradicate the tumor (122). In cervical cancer, similar numbers of immature DCs were found in tumor tissue as compared to healthy cervix (10, 120). The number of mature DCs was increased in tumor tissue, which may indicate that DCs may become activated in the tumor, but have decreased capacity to migrate out of the tumor. Alternatively, the observed number of DCs reflects a snap-shot of a population of DCs, which are preparing to migrate out of the tumor. In the tumor draining lymph node, IDO expressing DCs have been found (107), indicating that they may play a role in immune escape.

Myeloid derived suppressor cells (MDSCs) represent a heterogeneous population of incompletely differentiated myeloid cells (98). Their characterization is difficult due to the complicated phenotype. They are generally characterized as CD11b+CD14-, CD33+HLA-DR- or CD14+HLA-DR- (123, 124). They are elevated in the peripheral blood of cancer patients (reviewed in (123)). MDSC tumor infiltration has not been studied extensively, but MDSCs have been shown to infiltrate hepatocellular and head and neck carcinoma (125, 126). These MDSCs are able to directly inhibit T-cell responses via ROS and iNOS (127). In mice these cells promote tumor progression (128). Their impact on tumor progression in cancer patients is still unclear.

Even though the DC, TAM and MDSC populations are described as separate entities above, they all are from myeloid origin and therefore they are derived from the same precursors. As a result, there may be a spectrum between these populations in which single cells may have characteristics from multiple cell populations.

4.4. The role of adaptive T regulatory cells in immune evasion

CD4+ Tregs have emerged as arm of the adaptive immune response involved in counteracting the anti-cancer response. Tregs regained the interest of the field of immunology due to the fact that they display a high expression of CD25 and depletion of these CD25 positive immune cells led to autoimmunity in mice (129). Furthermore, CD25 depletion enhanced the immune response to TAA in mouse tumor models (extensively reviewed in (130, 131)). In cancer patients, increased levels of CD4+CD25^{high} Tregs have been detected in the peripheral blood, in the tumor microenvironment and in tumor-draining lymph nodes. These human CD4+CD25^{high} T cells display suppressive capacity in vitro (132-134). Strategies to deplete CD4+ Tregs on the basis of their CD25 expression have been translated to the clinic (135). In some, but not all studies an improved anti-tumor response was achieved (130). Moreover, a recent study showed that depletion of natural occurring Tregs may enhance tumor vaccine efficiency (136), underlining the importance of Tregs in dampening immunity against cancer. Recent studies revealed that CD4+ Tregs can specifically recognize TAA and TSA in mice and human, which will be discussed below.

Besides CD4+ Tregs, CD8+ Tregs have also been implicated to play a role in cancer patients, as plasmacytoid DCs isolated from ovarian cancer ascitis stimulated the outgrowth of CD8+ Tregs (137). From the majority of a large series of different human cancers, functionally active tumor-infiltrating CD8+CD28- Tregs could be isolated which exerted their suppressive effect via the secretion of IL-10 (138, 139). Furthermore, tumor-infiltrating contact-dependent CD8+CD25+Foxp3+ Treg clones were obtained from prostate cancer patients (140). In addition, CD3+CD56+CD8+ T cells with suppressor activity were observed in the blood of patients with colorectal cancer (141). Except for CD8+ Tregs which recognized the oncofetal protein expressed by breast carcinoma in humans (139) and RFM lymphoma in mice (142), it is not known which antigens are recognized by CD8+ Tregs. Their impact on patient survival is also still unknown.

4.4.1. Clinical impact of tumor infiltrating CD4+ Tregs

There is a large body of evidence indicating a role for tumor-infiltrating Tregs in the clinical outcome of cancer patients. An early study in colorectal cancer showed that patients with a higher tumor-infiltrating CD8+/CD4+ T-cell ratio, as determined by immunohistochemistry, displayed an improved prognosis (143). This implied that co-infiltrating CD4+ T cells were associated with a less effective anti-tumor response and that they may even dampen the anti-tumor response. While the use of CD25^{high} is a suitable marker for Tregs by flowcytometric analysis, it cannot be used in immunohistochemistry because CD25 is also expressed on activated CD4+ T cells and it is impossible to distinguish them from CD4+ Tregs. Identification of FOXP3 as intracellular marker for CD4+ Tregs put this field forward (144-146). FOXP3 is one of the proteins identified as essential for the development and function of so-called natural Tregs (144, 147). Another protein is the Wiskott-Aldrich syndrome protein which regulates homeostasis and peripheral activation, expansion and function of natural Tregs (148-150). Tumor-infiltrating FOXP3+ T cells have been studied extensively. In different types of cancer, increased numbers of tumor-infiltrating FOXP3+ immune cells are associated with a poorer prognosis (151-156). In head and neck cancer and follicular lymphoma, the number of infiltrating FOXP3+ immune cells was even found to be an independent prognostic factor (157, 158). In cervical cancer, ovarian cancer, cutaneous T-cell lymphoma and lung cancer, the effect of FOXP3+ immune cells on prognosis was more pronounced when the ratio between CD8+ and FOXP3+ T cells was calculated (10, 15, 159-161). More extensive studies showed that the balance between tumor-infiltrating CD8+ and CD4+ FOXP3+ T cells could serve as an independent prognostic factor in hepatic carcinoma, Hodgkin's lymphoma and also in cervical cancer (15, 162-164). While it is clear that in vivo the presence of CD4+ FOXP3+ T cells is associated with a dominant negative effect on the anti-tumor response, the expression of FOXP3 alone is not enough to mark human T cells as Tregs. One report revealed that transient FOXP3 expression could be observed in vitro on non-regulatory hyporesponsive CD4+ T cells while prolonged expression was found in CD4+ Tregs (165). Another study showed that naïve human T cells stimulated with plate-bound anti-CD3 and anti-CD28 could upregulate FOXP3 when cultured in the presence of TGFβ, but this did not convert them into Tregs in vitro (166). Moreover, the FOXP3-expression in these cells was lower than the expression observed in the CD25hi population, indicating that the levels are too low to convert them to a suppressive phenotype. In contrast to the in vitro induced FOXP3+ cells, the FOXP3+ cells isolated from tumor tissue do contain suppressive capacity and are therefore Tregs (167). Notably, not all tumor-infiltrating regulatory T cells express FOXP3 (168-170), indicating that there are different subsets of Tregs. Moreover, the presence of different subsets indicates that the number of tumor-infiltrating Tregs might be underestimated when studied through the expression of FOXP3. These studies collectively suggest that an analysis of the different subsets of T cells in tumor biopsies can be a valuable tool to predict clinical outcome and might be used to select patients for different types of immunotherapy treatment in the future as well.

4.4.2. Specificity of tumor-associated regulatory T cells

To date there is only limited knowledge on the origin and true specificity of CD4+ Tregs that infiltrate tumor masses. Since no lineage specific markers do exist, it remains a challenge to identify the true origin of these tumor resident Tregs. When the Tregs recognize TAA they might very well be primed in the thymus, however they can also be generated in the periphery from naïve precursors as was shown in a mouse colorectal cancer tumor model (171). Importantly, both types of CD4+ Tregs contribute to tumor-specific tolerance (172). The proof that CD4+ Tregs specifically recognize tumor-derived antigens comes from mouse studies in which tumor cells with model antigens are used in combination with the injection of TCR-transgenic naïve T cells which are specific for this model antigen (172, 173). Treg specific for the self-antigens LAGE-1 and ARTC1 have been found to be present among melanoma infiltrating lymphocytes (174, 175). These Tregs have a phenotype similar to thymus derived Tregs in terms of FOXP3, GITR, CTLA-4 and CD25 expression and cytokine production. More recently, Tregs specific for the TAA melanoma antigens gp100, TRP1, NY-ESO and SURVIVIN have been found in the peripheral blood of melanoma patients (177). While it is likely that these Tregs are primed in the thymus, it cannot be excluded that these Tregs were primed in the periphery during carcinogenesis. The nature of TSA dictates that TSA-specific Tregs must be induced in the periphery. Examples for the actual induction of TSA-specific Tregs are well studied in mice. Mice challenged with a tumor containing the TSA hemagglutinin (HA) mounted HA-specific CD4+ Tregs as well as HA-specific T-helper 1 cells (172), indicating that under specific conditions Treg are induced in the periphery as well. It is very well possible that Tregs specific for the HPV oncogenes are also present in tumors of cervical cancer patients.

5. Scope of this Thesis

Cervical cancer is induced by HPV in virtually all cases, but it is still unknown how these virus-positive tumors arise in the face of immunity. In this thesis we examined the role of different arms of the adaptive immune response on cervical cancer. In previous studies, it was shown that cancer patients do have a detectable immune response against the HPV16 E6 and E7 antigens. However, in general this response was not associated with cytokine production. In the present study, the mechanism underlying this dysfunctional immune response was investigated by studying the presence, specificity and function of the local T-cell response in patients with HPV-induced cervical malignancies at different stages of disease.

In Chapter 2, we assessed the impact of different types of infiltrating lymphocytes on the prognosis of cervical cancer patients. Infiltration of CD8+ T cells was found to be strongly correlated with improved prognosis. Moreover, the patients with better prognosis had a higher CD8+/CD4+ and CD8+/FOXP3+ ratio, whereas patients with a less favorable prognosis had a low CD8+/CD4+ and CD8+/Foxp3+ ratio. These results indicate that Tregs are able to hamper the CD8-mediated anti-tumor response.

The presence of HPV16-induced tumor-infiltrating T cells does not necessarily mean that these T cells also recognized the virus-derived antigens. To examine the specificity of tumor-specific infiltrating T cells, the presence of HPV16/18 E6/E7 specific T cells in tumor tissue and their draining lymph nodes was analyzed (Chapter 3). HPV16/18 E6/E7 specific CD4+ and CD8+ T cells were detected in 35% of the tumor biopsies, in 43% of the CIN biopsies and in 100% of the tumor-draining lymph nodes analyzed. Remarkably, most HPV-specific CD4 T cells were restricted by HLA-DQ and HLA-DP, which is in contrast to the HLA-class II expression pattern reported in cervical tumors (178).

Since we found that FOXP3+ T cells had a negative effect on the prognosis of cervical cancer patients, we investigated whether the HPV-specific tumor infiltrating CD4+ T cells were in fact Tregs. Since functional analysis is still the only way to truly identify Tregs, we analyzed the suppressive potential of the tumor-associated HPV-specific CD4+ T cells in an antigen dependent manner (Chapter 4.1). We found that HPV-specific Tregs were present in the tumor and in the tumor draining lymph node. These immune suppressive cells can potentially be boosted in cancer patients receiving therapeutic peptide vaccination. As part of a larger study, the impact of vaccination on the expansion of tumor-specific Tregs was assessed. We analyzed FOXP3+ and FOXP3- HPV-specific T cells in peripheral blood of patients before and after vaccination (Chapter 4.2). In most patients the HPV-specific CD4+FOXP3- T cells were boosted and/or induced after peptide vaccination. However, in some of the patients, similar numbers of CD4+FOXP3+ and CD4+FOXP3- T cells was induced and/or boosted by peptide vaccination. These results show that boosting of Tregs can occur after peptide vaccination and should not be neglected in vaccination strategies.

In Chapter 5 we investigated the polarization of the immune response in patients with premalignant lesions. HPV16-specific T-cell responses in HSIL patients were detected predominantly in patients who returned to the clinic for repetitive treatment of a persistent or recurrent HPV16+ HSIL lesion. Notably, these responses were not accompanied by the production of pro-inflammatory cytokines and thus indicating a dysfunctional T-cell response. Furthermore, we detected HPV16E7-specific Tregs in CD4+ T-cell bulk cultures isolated from HSIL tissue. These findings indicate that an immunosuppressive T-cell response can already occur at the early phases of malignant transformation.

HPV causes a chronic infection and subsequent cancer. It is unclear whether adaptive Tregs are also induced during acute viral infections. HPV particles are hard to culture in vitro so only low amounts of live virus can be obtained. Therefore it is impossible to study HPV-specific Tregs in the context of a viral infection.

To investigate whether Tregs are induced as a result of acute viral infections and whether these Treg can exert their effector function upon challenge with live virus, we studied the influenza specific response in healthy donors (Chapter 6). We detected circulating influenza-specific T cells displaying a similar IFNγ and IL-10 producing phenotype as previously observed for HPV16 in patients with cervical cancer. Moreover, isolated IL-10 producing T-cell clones were clearly able to suppress the function of both CD4+ and CD8+ T cells. Additionally, the influenza-specific Tregs were able to perform their suppressive function when challenged with live influenza virus infected cells. We hypothesize that during last phase of viral clearance, these T cells are required to dampen the immune response in order to prevent immunopathology. Importantly, this study showed that virus-specific Tregs can arise in protective immunity after acute viral infections.

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