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ORIGINAL PAPER

Immunosuppressive Tumor Microenvironment in Cervical Cancer Patients

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Abstract Cervical cancer is caused by Human papilloma-virus (HPV) in virtually all cases. These HPV-induced cancers express the viral oncogenes E6 and E7 and are therefore potentially recognized by the immune system. Despite the abundant presence of these foreign antigens, the immune system is unable to cope with the tumor. Due to the constant immunological pressure, cervical cancers can evolve different immune evasion strategies, which will be described in the current review. Several approaches for immunotherapy of cervical cancer are currently under development, which aim at inducing strong HPV-specific immunity. Besides the reinforcement of potent anti-tumor immune responses, immunotherapy could also enhance HPV-specific T regulatory cells. Supplementary strategies that neutralize an immunosuppressive milieu may have great potential. These strategies are discussed as well.

Keywords Cervical Cancer · Human papilloma virus · Immune response · Immune evasion · T regulatory cell

Abbreviations

HPV	Human papilloma virus
LSIL	Low-grade squamous intraepithelial lesion
HSIL	High-grade squamous intraepithelial lesion
IFNγ	Interferon γ
IL-5	Interleukin 5
CxCa	Cervical cancer
CTL	Cytotoxic T lymphocyte
NK cell	Natural killer cell
cFLIP	FLICE-inhibitory protein
IDO	Indoleamine 2,3-dioxygenase
VEGF	Vascuar endotheial growth factor
TGFβ	Tumor growth factor β
Tregs	Regulatory T cells
IRF	Interferon regulatory factor
TAM	Tumor-associated macrophage
DC	Dedritic cell
MDSC	Myeloid derived suppressor cells
PD1	Programmed death 1
APC	Antigen presenting cell

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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 [1–3]. 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 cases as well [4].

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 [5, 6] (Fig. 1).

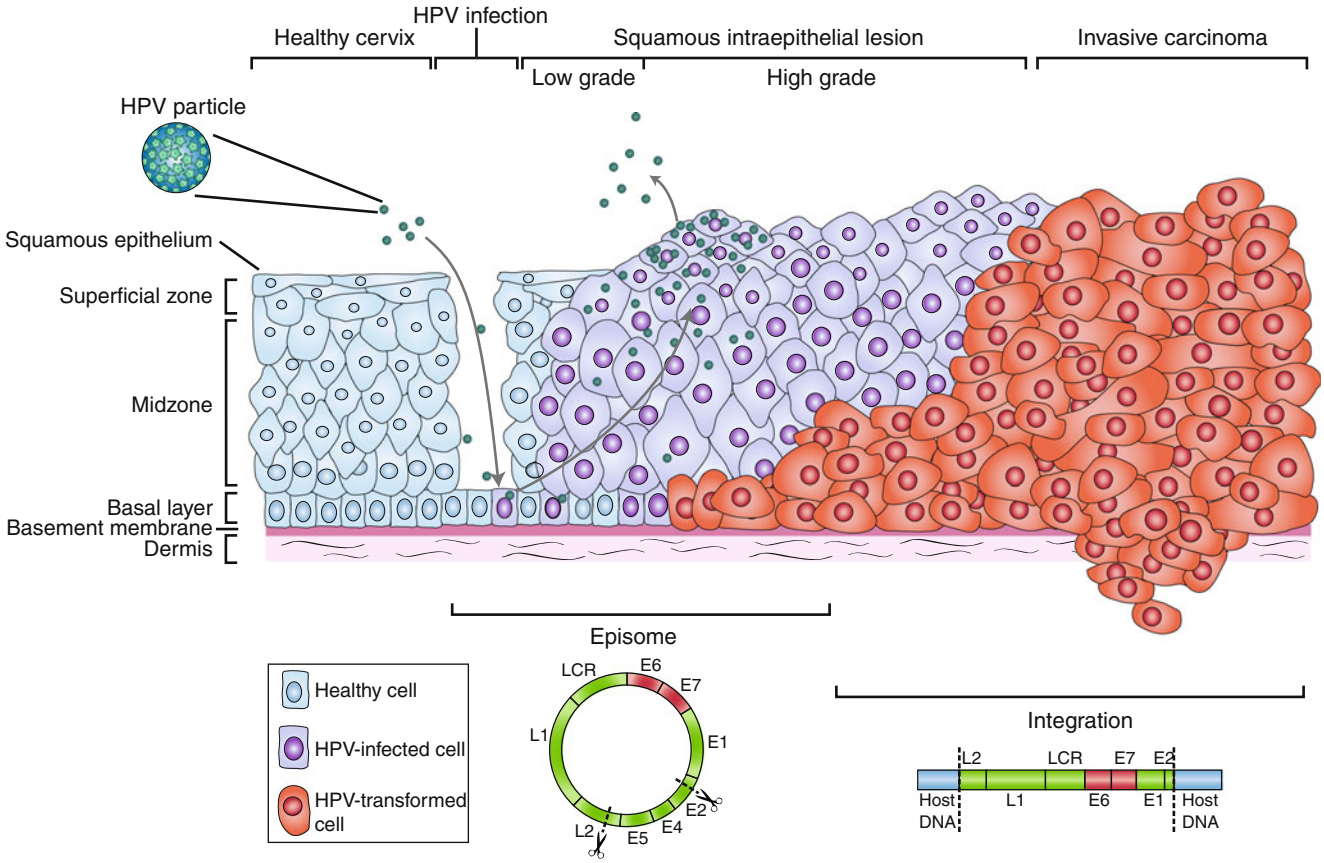

Fig. 1 Human papilloma virus (HPV)-induced malignant progression. Infection with HPV likely occurs in the basal layer of the cervical epithelium, which is exposed in a microlesion. During the productive lifecycle the early genes (E1, E2, E4, E5, E6 and E7) are expressed and viral DNA is replicated from episomal DNA. Hereafter, in the upper layers the late genes L1 and L2 are expressed and viral particles are assembled. Subsequently the virions are shed and new infection can be commenced. Low-grade squamous intraepithelial lesion (LSIL) support the production of viral particles. In a minority of infected women the lesion progresses into high-grade squamous intraepithelial lesion (HSIL). Progression towards microinvasive and invasive carcinoma is associated with the integration of the viral genome into host DNA and is frequently accompanied by loss of part of the viral genome, including disruption of the E2 gene. As a result, expression of the viral oncoproteins E6 and E7 are upregulated. LCR, long control region. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Woodman et al. [130]), copyright (2007)

The properties of both E6 and E7 are essential for HPV-induced malignant transformation and are therefore known as viral oncogenes [7]. 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 [8, 9]. Additionally, E6 can also promote the activation of telomerase [10]. E7 on the other hand is able to interact with the retinoblastoma family members and thereby it enhances cell proliferation [11]. Moreover, E7 stimulates cyclin A and E as well, promoting G0/G1 progression [12].

The progression of HPV infection to cervical cancer is a slow process and can be divided in 4 stages (Fig. 1). 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. 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 second 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 (third stage). The HSIL can progress further into the last stage, invasive carcinoma (reviewed in [13]). During the first 2 stages, spontaneous regression and/or clearance are common. It has been estimated that less than 1% of the infected women develop cervical cancer [14, 15]. 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 [16]. Additionally, early studies suggested that less than 30% of HSIL progress further into invasive carcinoma within 10 years [17].

During malignant transformation, the DNA of HPV is able to integrate into the host genome at random positions [18, 19]. The integration of the viral DNA is associated with the transition into invasive carcinoma [20]. 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 [5, 21]. Moreover, E6 and E7 are essential in maintaining the malignant phenotype of the tumor cells and are therefore expressed in every tumor cell [22]. Consequently, HPV E6 and E7 represent potential good targets for the immune system.

Immune Responses in Cervical Cancer Patients

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 IFNγ and IL-5 production and low levels of IL-10 [23–25]. Similar findings have been described for HPV18 [26]. Occasional responses against E7 are observed as well [24, 27]. T-helper responses against the C-terminal domain of HPV16E2 frequently occur at the time of virus clearance [28]. A recent prospective study showed that presence of HPV16 E2 specific T-cell responses were correlated with the absence of progression in LSIL patients, indicating a protective effect of E2-specific immunity [29].

In contrast, in cervical cancer patients HPV-specific T-cell responses are detected only in half of the Cervical Cancer (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 IFNγ, but the anti-inflammatory cytokine IL-10 was still detected in CxCa patients [25, 30]. Similar results were found for HPV18 as well [26]. 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 [31–34]. The presence of HPV16 E6-specific responses in CxCa patients are associated with invasion depth and are associated with disease free survival [35]. HPV16-specific CTL can only rarely be detected in the peripheral blood of HSIL and CxCa patients [36–39], whereas such responses are frequently detected in healthy donors [40, 41]. Since CD4 T cells are essential in the induction and maintenance of CD8 cytotoxic T-lymphocyte (CTL) immunity [42], 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.

HPV-specific T cells have been reported to infiltrate cervical neoplastic tissues and metastatic lymph nodes as well [43–47]. These infiltrating T cells are not specific for preferential regions within the E6 and E7 proteins. Remarkably, most of the CD4 restricted T-cell responses were restricted by HLA-DP [43]. This might be specific for HPV-induced tumors, but warrants further investigation. Within a single patient the HPV-specific T-cell response is broad as is indicated by the recognition of multiple E6 and E7 epitopes and multiple T-cell receptor Vβ usage [48].

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 [49, 50]. 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.

Despite the abundant presence of HPV-specific T cells in neoplastic tissue, the immune system is unable to eradicate the tumor. This suggests the existence of an immunosuppressive microenvironment in cervical cancer patients.

Immune Evasion Strategies Employed by Cervical Tumors

During malignant transformation, a continuous struggle exists between the tumor cells and the immune system. Because of continuous immunological pressure, the tumor develops several mechanisms to escape immunosurveillance. As the tumor persists it may accumulate such mechanisms, thereby evading control by the immune system. This is a slow process that can take years or even decades and is known as cancer immunoediting [51]. In many tumors the transformed cells have acquired several mechanisms to protect them from immune cell mediated killing. These mechanisms include (A) 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 (Fig. 2). The different mechanisms described in the literature and their role in cervical cancer will be discussed below.

Direct Evasion of the Anti-Tumor Response

The occurrence of antigen loss has been well demonstrated in an immunogenic tumor mouse model [52, 53]. These studies collectively show that tumor cells are able to lose

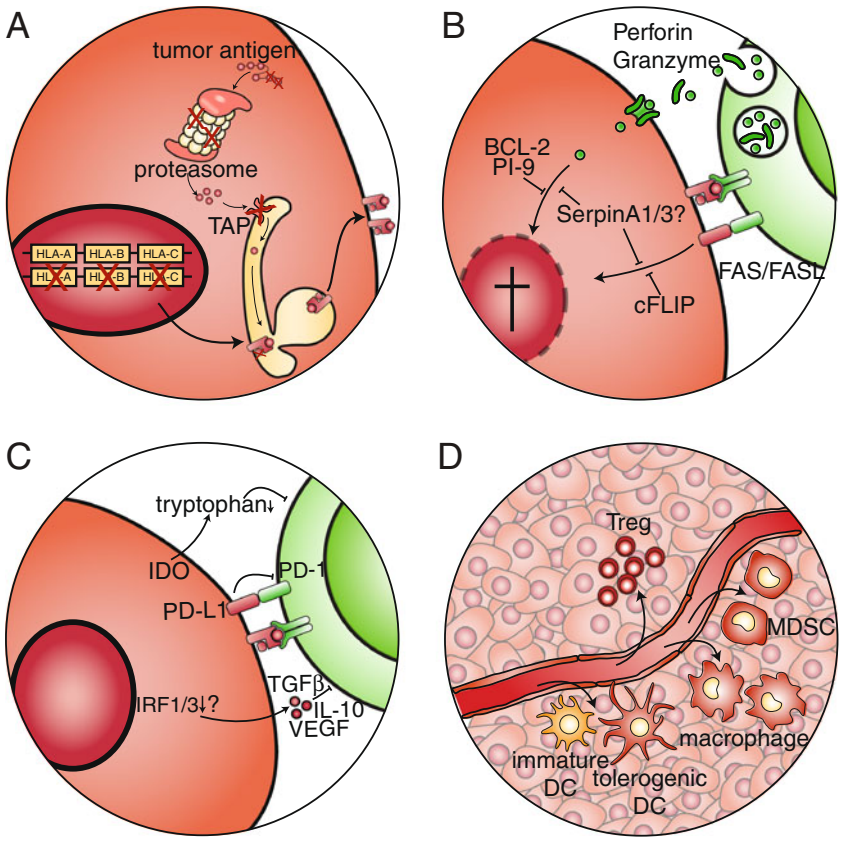

Fig. 2 Immune evasion mechanisms employed by tumors. In many tumors the transformed cells have acquired several mechanisms to protect them from immune cell mediated killing. These mechanisms include **a** downregulation of the antigen presentation machinery, **b** insensitivity to CTL-mediated cytotoxicity, **c** production of immunosuppressive cytokines and **d** attraction of immune cells with immunosuppressive properties

the expression of antigens as a result of immunological pressure. Occurrence of antigen loss has also been illustrated in melanoma patients. Antigens normally expressed by melanocytes are frequently lacking in tumor cell lines and tumor tissue from melanoma patients (reviewed in [54]). 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 [1, 55, 56]. 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 [57]. For these apoptotic pathways, tumor-escape variants have been described (reviewed in [58]). Examples of such escape-mechanisms are overexpression of the anti-apoptotic gene BCL-2 [59], expression of the FASL-inhibitors FLICE-inhibitory protein (cFLIP) [60, 61] and expression of the Granzyme B inhibitor PI-9 [62] in mouse models. cFLIP has been shown to be overexpressed in cervical tumor tissue compared to healthy cervix, but the impact on survival is still unclear [63]. 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 [64]. Since SerpinA1 and SerpinA3 both have been implicated in inhibition of apoptosis [65, 66], 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 [67]). Mutations of the individual HLA alleles together with the deletion of the common β2 microglobulin genes are commonly observed in many types of cancer. A different immune escape mechanism employed by a number of tumors is defects in the antigen processing machinery. Defective antigen processing leads to impaired antigen presentation of tumor antigens, as a result viral and tumor associated antigens normally produced by the proteasome and transported through TAP cannot be presented on MHC class I [54]. 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 [68]. Since antigen presentation of the HPV16E6 protein depends on TAP and the proteasome [69], 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 [70, 71], 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 MHC class I heavy chain, LMP2 and/or TAP1 [72]. 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 [73]. 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 [74]. 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 [75, 76]. 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 transformation, due to immunological pressure on the tumor. In cervical cancer patients abnormalities in the MHC class I presentation machinery has been well documented [49, 74–82]. Alterations in MHC-class I presentation pathway has been observed in approximately 90% of the patients with cervical cancer [79]. 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.

Indirect Evasion of T-Cell Mediated Killing

Many tumors express inhibitory coreceptors [83]. The inhibitory B7 family member B7-H1 (PD-L1) is expressed on a wide variety of tumors [84]. This molecule can interact with PD-1 and CD80 on T cells, thereby inducing apoptosis, anergy or exhaustion of effector T cells [85, 86]. In a variety of tumors, expression of PD-L1 is associated with poorer survival [87–94]. Unexpectedly, expression of cell-surface PD-L1 in cervical cancer patients was associated with improved survival [95]. This phenomenon could be explained by incapacitation of infiltrating PD1+ regulatory T cells (Tregs) through PD1:PD-L1 interactions. 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 [96, 97], but their role in cervical cancer is unknown.

Immunosuppressive factors produced by tumor cells can also contribute to the immunosuppressive microenvironment. These factors include indoleamine 2,3-dioxygenase (IDO), vascuar endotheial growth factor (VEGF), tumor growth factor β (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 [98]. A few studies showed that IDO is expressed by the tumor and the level of expression is an independent prognostic factor in colorectal cancer [99, 100]. IDO has been implicated to interfere with the initial immune response to tumor antigens, the cytolytic capacity of CTL and enhanced suppressive capacity of Tregs in several tumor types (reviewed in [101]). IDO has been shown to be present in HSIL and CxCa, but the functional consequence of IDO was not addressed [102]. 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 [103]. TGFβ is expressed in many tumors and is known to inhibit immune responses at multiple levels [104, 105]. In cervical tumors, TGFβ mRNA is frequently detected but does not correlate with survival [106]. However, PAI-1 and αvβ6 integrin expression, which reflect the presence of active TGFβ, has a negative influence on survival [106, 107]. Next to immune regulation, TGFβ also modulates other processes, which include cell invasion and metastatic colonization. The impact of TGFβ on immune escape is therefore difficult to determine in cancer patients [104]. 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 [108]. 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 [109, 110]. 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 [111].

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 antitumor responses. Macrophages are recruited by many types of tumors in high numbers, in these tumors they differentiate predominantly into a M2 phenotype [112, 113]. 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 [113]. Tumor infiltrating CD68+ macrophages have been found to infiltrate cervical tumors and metastatic lymph nodes [80, 114, 115]. The TAMs reach numbers similar to infiltrating T cells in cervical tumors [80]. However, the type of macrophage and their impact on the immune system have not been addressed in these studies.

Dendritic cells (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 [116]). 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 [117]. In cervical cancer, similar numbers of immature DCs were found in tumor tissue as compared to healthy cervix [50, 115]. 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 tumor draining lymph nodes of cervical cancer patients, IDO expressing DCs have been found [102], indicating that they may play a role in immune escape.

Myeloid derived suppressor cells (MDSCs) represent a heterogeneous population of incompletely differentiated myeloid cells [83]. Their characterization is difficult due to the complicated phenotype. They are generally characterized as CD11b+CD14-, CD33+HLA-DR- or CD14+ HLA-DR- [118, 119]. They are elevated in the peripheral blood of cancer patients (reviewed in [118]). Even though MDSCs in tumors has not been studied extensively, these cells have been shown to infiltrate hepatocellular- and head and neck carcinoma [120, 121]. MDSCs are able to directly inhibit T-cell responses via ROS and iNOS [122], and in mice these cells promote tumor progression [123]. Their impact on tumor progression in cervical cancer patients is, however, still unclear.

Even though the DC, TAM and MDSC populations are described as separate entities above, they all are of myeloid origin and therefore 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.

T Regulatory Cells in Cervical Cancer Patients

CD4+ Tregs have emerged as an arm of the adaptive immune response involved in counteracting the anti-tumor immune response. Early studies showed increased numbers of Treg, based on CD25 and CD152 expression in proximal tumor draining lymph nodes and these cells contained suppressive capacity [124]. Although Tregs can only be identified based on suppressive function, the transcription factor FOXP3 is currently the most widely used marker for Tregs [125]. The infiltration of tumors with FOXP3+ Tregs is unfavorable for patient survival in many types of cancer (reviewed in [126]). In cervical cancer patients the effect of FOXP3+ Treg was more pronounced when the ratio between infiltrating CD8+ T cells and FOXP3+ Tregs was calculated [49, 50]. The balance between infiltrating CD8+ T cells and Tregs was found to be an independent prognostic factor for patient survival [49]. Metastatic tumor cells in tumor draning lymph nodes were found to correlate with increased numbers of Treg in the respective lymph node [127].

As HPV-induced tumors express the viral antigens E6 and E7, the infiltrating Tregs potentially encompass HPV-specific Tregs. Indeed, Tregs specific for the E6 and E7 antigens have been detected in tumor- and HSIL infiltrating lymphocytes as well as tumor draining lymph nodes [30, 128]. Interestingly, these Tregs included both FOXP3+ and FOXP3- cells [126, 128]. This observation indicates that enumeration of Tregs on the basis of FOXP3 expression likely underestimates the total number of infiltrating Tregs in cervical tumors.

It is difficult to determine the true origin and role of HPV-specific Tregs during disease progression in cervical cancer patients. Possibly, Tregs are induced as part of the normal immune response against HPV, as acute infections such as influenza can mount virus-specific Tregs as well [129]. Generally, HPV infections are cleared quite slowly (median of 6 months) [130], while acute viral infections such as influenza are cleared within weeks. Therefore, the immune system seems to be inefficient in clearing HPV infections. This may be caused by early interactions of the host with the virus at multiple levels. Firstly, Langerhans cells, which are the professional antigen presenting cells in initiating mucosal immune responses, are improperly activated upon encounter of L2-containing virus like particles [131]. Secondly, HPV also interferes with the IFN pathway in infected keratinocytes, caused by the oncogenes E6 and E7 (Reviewed in [132]). This results in a stronger immunosuppressive microenvironment and may thereby promote the induction and expansion of HPV-specific Tregs. One or combinations of these interactions may result in enhanced induction of HPV-specific Tregs and as such induce a more immunosuppressive virus-specific immune response compared to acute viral infections. However, these observations do not explain why

most people are able to clear persistent HPV infections, whereas a minority of the infected women are not able to cope with the virus and as a result develop cervical cancer. Both genetic and environmental factors have been implicated in HPV oncogenesis, however a clear picture is still missing [133].

Accumulating numbers of circulating Tregs (defined as CD4+CD25+) have been detected in the peripheral blood of HSIL patients as witness of an immunosuppressive milieu in these patients [134, 135]. In line with these findings, a substantial number of infiltrating FOXP3+ Treg have been detected in HSIL patients [136, 137], but no significant differences were observed between HSIL and LSIL [137]. Moreover, HPV-specific Tregs were detected among cervical infiltrating lymphocytes in a patient with HSIL [30]. This is indicative of an immunosuppressive HPV-specific response in this patient. The immunosuppressive microenvironment in HSIL patients may subsequently favour the progression towards invasive carcinoma by evading immunosurveillance.

Additionally, HPV-induced tumor cells overexpress different self-antigens as well, including hTERT and p16 [138–141]. For this reason it is likely that Tregs specific for these antigens also infiltrate cervical tumors and contribute to the establishment of an immunosuppressive microenvironment in the tumor.

Immunotherapy of HPV-Induced Malignacies

Many different strategies have been developed for the immunotherapy of cancer [142]. Strategies against HPV-induced malignancies include synthetic long peptide vaccines, targeting the E6 and E7 proteins (reviewed in [42]). Although these therapeutic vaccines are designed to enhance CD4+ and CD8+ T-cell effector immunity, they may also activate pre-existing tumor antigen-specific FOXP3+CD4+ Tregs present in the lymph nodes and tumors of both cervical cancer and melanoma patients [143, 144]. In mice, boosting of Tregs after therapeutic vaccination was associated with subsequent failure of the anti-tumor immune response [145]. A recent study in vulvar intraepithelial neoplasia patients showed both clinical and immunological responses after vaccination against HPV16 E6 and E7 [146]. In this study patients who did not display a complete clinical response, mounted both HPV-specific effector T cells and HPV-specific FOXP3+ Tregs following vaccination. In contrast, patients who displayed a complete clinical response mounted predominantly HPV16-specific T effector cells [147]. These data indicate that those patients in whom the current HPV-specific therapeutic approach is unsuccessful could benefit from an alternative therapy that includes the neutralization of Tregs (Fig. 3).

Intervention Strategies to Bypass Vaccination-Induced Treg Expansion

Depletion of Treg Based on CD25 Expression

In several mouse models, treatment with an anti-CD25 depleting antibody enhanced the anti-tumor immune response (reviewed in [148]). For translation to the clinic a hybrid molecule has been used (ONTAK). This molecule contains full-length IL-2 for binding to CD25 and the translocation and toxic domains of diphtheria toxin to induce apoptosis [149]. In mice this molecule was able to deplete FOXP3+ Tregs in different compartments and was able to enhance vaccination-induced T-cell responses [150]. In combination with vaccination, ONTAK is able to deplete Tregs and thereby boosting the tumor-specific immune response in renal cell carcinoma, CEA-positive and melanoma patients [151–153]. In contrast, in one study ONTAK was unsuccessful in depleting Tregs in metastatic melanoma patients [154]. Together, these studies show that ONTAK as supplementary therapy in vaccination trials may be promising, however caution is needed as this therapy is not always successful.

LMB-2 is another immunotoxin, which targets CD25. LMB-2 is a hybrid molecule consisting of *pseudomonas* exotoxin A and the Fv chain of anti-CD25 [155]. In a small human trial, LMB-2 was able to partially deplete Tregs, but no effect was seen on vaccine-induced responses in patients with metastatic melanoma [156]. Further studies are required to determine a potential additive effect of LMB-2 treatment and HPV vaccination strategies.

Depletion of Tregs Based on Cytotoxic Chemotherapy

Low-dose cyclophosphamide, which is a cytotoxic alkylating compound, reduces both the number of Tregs as well as their function in mice [157]. A recent study showed enhanced Treg depletion in the tumor when cyclophosphamide was used in combination with an agonistic anti-OX40 antibody. This regime induced hyper-activation and cell death in the Treg compartment [158]. In animal models, low-dose cyclophosphamide was able to enhance vaccine-induced anti-tumor responses [159, 160]. In humans, cyclophosphamide used as a single agent was shown to inhibit the Treg compartment, while the effector compartment was not negatively influenced [161, 162]. In cervical cancer patients Treg numbers were decreased after

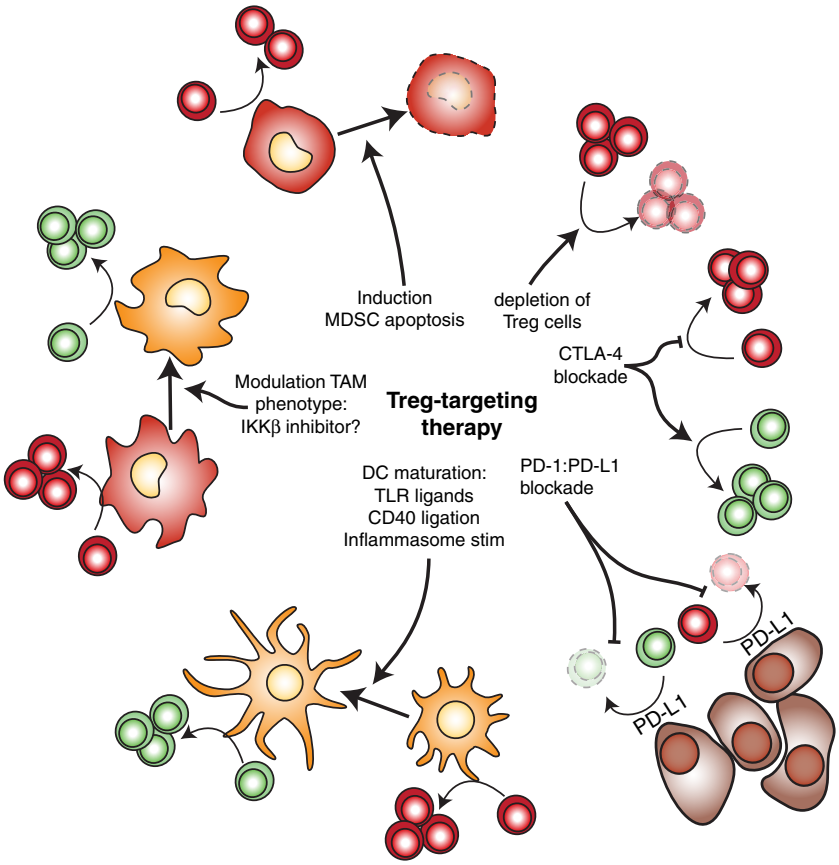

Fig. 3 Strategies that could bypass vaccination-induced Treg expansion. Depletion of Treg before or during treatment with CD25-targeting compounds or with low dose cyclophosphamide decreases the initial numbers of Treg. Blockade of CTLA-4 signaling both dampens Treg as well as releases the brakes on effector cells. Blockade of PD-1:PD-L1 interaction results in enhancement of effector responses, but also can enhance Treg function. Several agents can be used to skew the antigen presenting compartment to an immunogenic phenotype. These approaches include maturation of DC, modulation of macrophage phenotype and targeting myeloid suppressor cells (MDSC)

preoperative low-dose chemoradiation therapy [127, 163]. Combinational therapy has not been studied in cervical cancer patients, but may prove to be an effective approach to enhance anti-tumor vaccination strategies.

CTLA-4 Blockade to Improve Anti-Tumor Immunity

CTLA-4 is an inhibitory co-receptor that is expressed both on activated T cells and constitutively on thymus derived Tregs. In mouse models, it has been shown that combination therapy of CTLA-4 blockade, especially together with CD25 depletion or GM-CSF secreting vaccine improves immunotherapy against established tumors [164–166]. CTLA-4 blockade both on the effector population as well as on the Treg compartment is important in the enhancement of anti-tumor responses [167]. Two monoclonal blocking antibodies (ipilimumab and tremelimumab) are currently being tested in clinical trials [168]. Since these antibodies affect all T cells regardless of specificity, side effects of these antibodies include mild to severe autoimmunity [168, 169]. Early promising clinical trials show enhanced anti-tumor T-cell responses upon treatment with anti-CTLA4 antibodies [170–172]. A recent phase III trial showed increased survival in melanoma patients after ipilimumab treatment [173]. However, in this study treatment was not improved by gp100 specific vaccination. These monoclonal antibodies may provide a window in which CTLA-4 blockade combined with vaccination against HPV16 E6 and E7 may improve the treatment of cervical cancer patients.

Blockade of the PD-L1-PD1 Axis

Blockade of PD1 or PD-L1 improves anti tumor-responses in several mouse models (Reviewed in [174]). A humanized blocking antibody to PD-1 has been tested in a phase I trial in patients with hematological malignancies and was found to be well tolerated in these patients [175]. As PDL-1 expression in cervical cancer affects patient survival differently compared to other types of cancer, treatment with PD-L1 blocking antibodies may have unexpected results in cervical cancer patients.

Modulating Antigen Presenting Cells

Different subsets of APC have the capacity to induce Tregs [118, 176, 177]. As these cell types are not affected using the strategies described above, depletion of Tregs does not exclude *de novo* induction of HPV-specific Tregs upon tumor-specific vaccination. Therefore, strategies to modu-

late these cells as well may prove to be a valuable supplementary therapy to enhance tumor-specific immune responses.

Several approaches have been proposed to skew the phenotype of DCs in cancer patients from a tolerogenic into a pro-inflammatory phenotype (reviewed in [117]). These approaches include activation of DCs by anti-CD40 antibodies, Toll-like receptor ligands, activation of the inflammasome and immunogenic cell death by chemotherapy and radiation therapy [178–180]. In cervical cancer patients, these properly activated DCs may in turn shift the balance from a Treg dominated response into a Th1/CTL dominated HPV-specific response, which is subsequently able to mount a full-blown attack against the tumor.

Tumor associated macrophages promote the immunosuppressive microenvironment. Targeting these cells may therefore augment vaccination protocols. Two recent studies described that skewing of the phenotype towards a proinflammatory M1 phenotype by inhibition of IKKβ results in improved tumoricidal activity [181, 182]. The M1 macrophages in turn may promote anti-tumor immune responses. Even though subversion of the phenotype of macrophages represents a promosing approach for anticancer therapy, agents are not yet available to promote M1 macrophage differentiation in the clinic. However, a recent study in a mouse model of HPV-induced tumors showed that depletion of TAM by clodronate-containing liposomes impaired tumor growth in mice [183]. These strategies are still in preclinical models, but hold potential to improve therapeutic HPV vaccination.

Several agents are currently tested in preclinical models to inhibit expansion and function of MDSCs (Reviewed in [118]). These cells are implicated in the expansion of Tregs and are present in the peripheral blood of cancer patients in relatively high numbers. Therefore, depletion of MDSCs may result in abrogation of the immunosuppressive milieu, enabling effective vaccination against HPV E6 and E7 without vigorous expansion of Tregs.

Final Remarks

The local presence of an immunosuppressive microenvironment provides a plausible explanation for the inability of the immune system of cervical cancer patients to cope with the tumor. Moreover, HPV-specific Tregs are boosted upon vaccination with HPV16 synthetic long peptides and negatively correlate with clinical outcome. Therefore, elimination/reduction of the Treg compartment either before or during vaccination, will likely shift the balance from a Treg dominated response to an effector T-cell dominated response. This will result in improved vaccination efficacy. Strategies that elicit potent anti-tumor immune responses may also lead to the induction of different escape mechanisms. These mechanisms could include antigen loss, loss of MHC-class I molecules and impaired antigen processing. These escape variants can subsequently be targeted by alternative approaches, such as vaccination against epitopes that are associated with impaired antigen processing [184].

Finally, the tumor microenvironment observed in cervical cancer patients has similar characteristics to other types of cancer. Knowledge gathered on inducing a potent anti-tumor immune therapy in these patients may therefore be translated to other types of cancer as well.

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