Clinical implications of immunecell infiltration in vulvar intraepithelial neoplasia

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CHAPTER 2

TREATMENT FAILURE IN PATIENTS WITH HPV 16-INDUCED VULVAR INTRAEPITHELIAL NEOPLASIA: UNDERSTANDING DIFFERENT CLINICAL RESPONSES TO IMMUNOTHERAPY

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Expert Rev. Vaccines 2012 Jul;11(7):821-40

Abstract

A failure of the immune system to launch a strong and effective immune response to high risk HPV is related to viral persistence and the development of anogenital (pre)malignant lesions such as vulvar intraepithelial neoplasia (VIN). Different forms of immunotherapy, aimed at overcoming the inertia of the immune system, have been developed and met with clinical success. Unfortunately these, in principal, successful therapeutic approaches also fail to induce clinical responses in a substantial number of cases. In this review we summarize the traits of the immune response to HPV in healthy individuals and in patients with HPV induced neoplasia. We discuss the potential mechanisms involved in the escape of HPV-induced lesions from the immune system and indicate gaps in our knowledge. Finally, the interaction between the immune system and VIN is discussed with a special focus on the different forms of immunotherapy applied to treat VIN and the potential causes of therapy failure. We conclude that there are a number of pre-existing conditions that determine the patient's responsiveness to immunotherapy and that an immunotherapeutic strategy in which different aspects of immune failure are attacked by complementary approaches will improve the clinical response rate.

Keywords: VIN, immunotherapy, immune modulation, vaccination, HPV, immune escape, regulatory T-cells, macrophages

Introduction

Vulvar intraepithelial neoplasia (VIN) is a chronic vulvar skin disease with malignant potential that often causes severe and long-lasting complaints of pruritis, pain and sexual dysfunction.[1] In 2004, VIN lesions were classified according to the International Society for the Study of Vulvovaginal Disease into; usual type VIN (uVIN), historically called VIN 2 and 3, and differentiated type VIN (dVIN).[1,2] The incidence of dVIN accounts for less than 5% of all VIN lesions, occurs in older women, and is associated with chronic dystrophies of the vulva such as lichen sclerosus and lichen planus.[1-3] dVIN has a high malignant potential (5.6 fold compared to uVIN).[1,3] UVIN is caused by a persistent human papilloma virus (HPV) infection, in particular HPV type 16, which is present in over 90% of cases.[1,4,5] The incidence of uVIN, approximately two per 100,000 women, is increasing worldwide and is related to the increase of HPV infections in young women.[3,6-9] UVIN occurs predominantly in younger women (peak incidence 40 years), tends to be multifocal in 60% of patients and is correlated with smoking.[1,2] Progression rates to malignancy of uVIN are estimated at 3-4% after treatment and 9% without treatment in 1–8 years, whereas spontaneous regression of VIN occurs in less than 1.5%.[10,11] Treatments are therefore aimed at both relief of symptoms and prevention of progression into (micro-)invasive lesions. Conventional surgical treatment is often disfiguring, mutilating and suboptimal, as reflected by the high recurrence rates of 20-40% and physical and psychological morbidities.[10,12-14]

HPV is a DNA virus that infects the basal cells of the genital epithelia, in particular the squamous epithelium, and is the most common sexually transmitted pathogen worldwide.[9,15] Over 100 types of HPV are identified, which are subdivided into low risk (non-oncogenic; *e.g.* lr-HPV 6 and 11) and high risk HPV (oncogenic; *e.g.* hr-HPV 16 and 18)[16] Approximately 60% of young women are infected with either an hr-HPV (40%) or lr-HPV (20%) within the5-year period after they become sexually active, while the lifetime risk of acquiring an HPV infection is estimated at 80%.[8,9,17,18] In most cases the infection is asymptomatic and is cleared within 1 year.[8,17] Persistent infections only develop in less than 10% of the infected women and are causally related to the development of intraepithelial neoplasia of the cervix (CIN), vagina (VAIN), anus (AIN) and/or vulva (VIN) and their subsequent progression to invasive squamous- or adeno-carcinoma.[8,9,17,19,20] Multicentric disease affecting the cervix, vagina and/or anus have been described in 22–71% of VIN patients.[21,22] The risk factors associated with HPV-induced disease are the lifetime number of sexual partners, smoking (as it results in a decreased local immune response), and the use of oral contraceptives (of which the estrogens may increase cellular proliferation via an effect on the early oncoproteins of HPV).[23,24]

The early viral oncoproteins of HPV (E1, E2 and E4–E7) are the key factors in progression of HPV induced disease because they have different and synergistic functions in the

maintenance, replication and progression of a potential HPV associated lesion.[15,16,25,26] These early oncoproteins influence several signal transduction pathways such as the cell proliferation pathway due to inactivation of cell cycle arrest protein $p16^{INK4A}$, the TNF-α and IFN pathways.[26] The pivotal players E6 and E7 are constitutively expressed in malignant tissue and their expression results in enhanced cell proliferation and subsequent viral genome replication.[15] E6 downregulates p53 and expression of the proapoptotic protein BAK, leading to resistance to apoptosis and increased chromosomal instability.[25] E7 binds to and degrades the retinoblastoma susceptibility protein (pRB), which leads to apoptosis. By transcriptional activation of the cyclin A and cyclin E genes, E7 regulates cell proliferation and downregulates $p16^{INK4A}$, which can counteract the function of E6.[25] The E1 protein is essential in HPV replication where the E2 protein acts both as a replication and transcription activator.[16] E2 represses the viral promoter of E6 and E7 during early stages of infection.[27] Carcinogenic progression is accompanied by integration of the viral genome into the host cell DNA which disrupts E1 and E2 function and enables upregulation of E6 and E7 expression.[27] E5 appears to be important in the early course of infection by stimulating cell growth and preventing apoptosis following DNA damage.[25]

The high prevalence of hr-HPV infection in uVIN has lead to the suggestion that therapy should aim for the immunological eradication of virus-infected cells. Different types of local and systemic forms of immunotherapy have already been described with encouraging clinical results; in a number of trials almost half of the treated patients had durable complete lesion regression.[28-33] Notwithstanding these successes, these therapeutic approaches also fail to induce clinical responses in a substantial number of cases. The aim of this review is to provide insight into the nature of HPV-induced disease, to indicate the gaps in our knowledge of the interaction between uVIN and the immune system and to identify the possible causes of immunotherapy failure as a guide to optimize the immunotherapy of uVIN.

Immunity to HPV infection

Infection with HPV occurs when the epithelial surface is disrupted through minor damage of the genital mucosa, thereby allowing access to the basal cells of the epithelium.[25] Here, the early proteins E6 and E7 are expressed.[15] In the suprabasal layers, E1, E2 and E5 are expressed and viral replication takes place.[15] In the most superficial layers, newly made viral DNA is encapsulated by the late structural proteins L1 and L2 and the new virions are released by wear and tear of the epithelial surface.[15] The absence of a cytopathic phase or systemic viraemia reduces the potential exposure of HPV to the immune system and causes delay in the activation of the immune system. This is not absolute as over 80% of the HPV infections are controlled within 2 years after infection.[8,17,34] There is strong evidence that both the innate and the adaptive arms of the immune system play a role in the protection against HPV as will be described in detail below.

Innate immunity

The innate immune system acts as the first line of defense against invading viruses. Keratinocytes express pathogen recognition receptors (PRRs), including the membrane bound toll-like receptors (TLR), the cytoplasmic NOD-like receptors (NLR) and RNA helicase retinoic-acid-inducible gene I (RIG-1) and melanoma differentiation-associated gene 5 (MDA5).[35-39] The latter two recognize double-stranded viral RNA in the cytoplasm and are constitutively expressed in human keratinocytes.[36-38] The TLR family (TLR 1-10) recognizes different molecular patterns; TLR3 recognizes double-stranded RNA, TLR7 and TLR8 recognize single-stranded RNA found during viral replication, whereas TLR9 recognizes unmethylated CpG motifs common in viral DNA.[35,36,40] TLR3 is expressed in undifferentiated keratinocytes while the expression of TLR9 in undifferentiated keratinocytes is debated or present at very low levels.[38-40] Both TLR3 and TLR9 are capable of regulating proinflammatory responses, whereas TLR7 and TLR8 were not functionally expressed in undifferentiated keratinocytes.[38-40] However, human keratinocytes are able to upregulate TLR7 in response to stimulation with poly I:C, which is a strong agonist for TLR3, RIG-I and MDA5, suggesting that under inflammatory conditions the keratinocytes may become responsive to immune-modifying TLR7 agonists.[37] Binding of viral components to these receptors during early stages of viral infection leads to direct NF-kappa-B activation, which results in upregulation of pro-inflammatory cytokines, and/or activation of type I interferon (IFN) response genes, including transcription factors IRF3 and IRF7 regulating the production of antiviral and pro-inflammatory cytokines (e.g. GM-CSF, IL-1β, TNF-α, IL-10, IL-12, MIP3α).[35-40] Proliferative cytokines and chemokines influence the migration and function of antigen presenting cells (APCs), with Langerhans cells (LCs) and dermal dendritic cells (DCs) being their main representatives in the skin.[41,42] This wide variety of PRRs present in human keratinocytes reflects their ability to respond to different classes of pathogens and HPV infected keratinocytes should be able to detect the presence of HPV genomic DNA directly via TLR9 or indirectly via RIG-I.[36,38]

Cellular immunity

The important role of the immune system in protection against HPV-induced lesions is demonstrated by the high incidence of persistent HPV infections and subsequent HPV-related malignancies in immunosuppressed individuals.[43,44] On the other hand, only a minority of infected non-immunosuppressed subjects develop progressing epithelial lesions or cancer.[8,9,17-20,45] Composite data indicate the importance of CD4+ T-cells in the control of HPV-induced disease as more severe lesions are observed in HIV+ patients with low numbers of circulating CD4+ T-cells.[45,46] In addition, the increase in CD4 cell count after anti-retroviral treatment correlates with the regression of HPV-induced CIN lesions in HIV+ patients.[46] Activation of the adaptive immune response is dependent on cross-presented

viral antigens by activated LCs and DCs. Depending on the different environmental cues in the microenvironment; the APC will adopt a certain state of differentiation and migrate to the local lymphoid tissues to present antigens to naïve T-cells. Depending on the status of the APC, as reflected by the levels of co-stimulatory or inhibitory molecules and its cytokine production (e.g. IL-12 or IL-10), a T-cell response will be induced that can consist of different types of CD8+ T-cells, CD4+ helper T-cells (Th-cells) and regulatory T-cells (Tregs).[47,48] At the time of spontaneous regression of HPV-infected genital warts, the lesions are infiltrated with CD8+ cytotoxic T-cells (CTL), CD4+ T-cells and macrophages.[49] Approximately 60% of the healthy subjects display a directly ex-vivo detectable type 1 (i.e. IFNγ associated) T-cell reactivity against the early oncoproteins E2, E6 and E7.[50,51] Spontaneous regression and clearance of HPV-induced lesions is associated with the presence of both lesion infiltration and circulating CD4+ and CD8+ T-cells directed against the early oncoproteins whereas this type of immunity is weak or lacking in patients with progressive HPV-induced diseases.[45,52-58] These data indicate that type 1 T-cell responses to the HPV 16 early proteins play an important role in the protection against persistent HPV infection.This notion is sustained by the data obtained from clinical trials in which the full regression of HPV 16-induced high-grade vulvar lesions is strongly associated with the presence of a proliferative or type 1 HPV-specific T-cell response prior to the treatment.[30,59] Moreover, clinical regression after immunotherapy by vaccination is associated with the strength (i.e. breadth and magnitude) of the vaccine-induced proliferative and/or IFNγ-associated HPV-specific T-cell response.[29,31,60,61] Notably, a directly ex-vivo detectable type 1 T-cell reactivity against the late structural antigen L1 is not only found in healthy subjects but also in the majority of patients with HPV-induced disease.[55] Furthermore, strong type 1 L1-specific T-cell responses are induced by vaccination with L1 virus like particles (VLPs), yet these vaccinations are not able to induce clearance of established infections.[55,62] Together these observations indicate that the response against L1 is not essential for T-cell mediated protection once a person is infected by hr-HPV.

Humoral immunity

Antibodies to the HPV viral capsids L1 and L2 can be detected from approximately 6 months post infection although 30-50% of patients with persistent infections never seroconvert.[63,64] Immunoglobulin-G (IgG) seroconversion rates appear to be higher among women with persistent infection over a long period of time.[65] In general, IgG antibody responses to HPV L1 and L2 are weak during infection (i.e. at low levels) and do not protect against re-infection with the same HPV type or clear HPV-induced lesions as discussed above.[66-68] However, the induction of high levels of antibodies to the virus capsid protein – via prophylactic vaccination with VLPs – prevents viral infection very efficiently and has led to the introduction of two commercial vaccines (Gardasil® and Cervarix®).[69-71] The

prophylactic vaccine protection times depend on follow-up in the clinical trials and range from 4 to 9 years.[69-71] However, no accelerated clearance of existing viral infections has been observed despite these high antibody levels.[62] Antibodies reactive to E6 and E7 are also frequently found in patients with HPV-induced cancer and their induction appears to be dependent on the clinical stage of disease, with approximately 20% of seroconverters at FIGO stage I up to more than 50% in stage III.[72,73] Particularly in early stage disease, the antibody response to HPV 16 E6 is more frequently found compared to E7.[72,73] While it does not appear to affect prognosis at the stage of cancer, positive humoral reactivity to E7 was observed in patients who had cleared the viral infection rather than patients with persistent infection.[74] Although these antibodies are not expected to exert any direct effect on infected or transformed cells, their presence indicates active priming of an underlying T-cell response. Notably, HPV-specific T-cell responses are also found more frequently in cervical cancer patients with more advanced stages of disease.[75]

Immunogenetics

Besides environmental and lifestyle factors, host genetic factors are likely to play a role in persistence and appearance of HPV-induced neoplasia. The antigen processing machinery (APM) and human leukocyte antigen (HLA) class I molecules are key in the presentation of antigenic peptides to CD8+ T cells and, therefore, are important in the destruction of virally infected or transformed cells.[76,77] Defects in the APM and HLA molecules thus may contribute to viral escape, persistence and ultimately induce malignancies. From an array of 13 non-synonymous coding single nucleotide polymorphisms (SNPs) in the *LMP2*, *LMP7*, *TAP1*, *TAP2*, and *ERAP1* genes, the allele distributions at the *LMP7-145*, *TAP2-651*, *ERAP1-12,* and *ERAP1-730* loci differed significantly between cases and controls with the major allele at the *LMP7* and *TAP2* loci and the minor allele at both *ERAP1* loci associated with increased risk for cervical carcinoma.[76,77]

Over 800 different HLA class I and class II alleles have been defined and it is possible that some HLA molecules may be more or less suitable to present HPV-derived peptides and as such influence the ability to clear an HPV infection or HPV-induced neoplasia.[78] Indeed, the susceptibility or resistance to HPV infection and HPV-induced lesions has been associated to particular HLA alleles, albeit that many of these findings were not consistent across different populations.[79] A protective effect of HLA class II DRB1*13/DBQ1*0603 alleles is the most consistently found association, although the effect was only significant in 47% of studies.[79] HLA-DRB1*07 and DRB1*15/DQB1*0602 have been associated with an increased risk of HPV-induced cervical neoplasia in The Netherlands and we confirmed that HLA-DRB1*07 was overrepresented in Dutch patients with HPV 16-positive cervical cancer whereas HLA-DRB1*13 was underexpressed in patients with cervical cancer compared to controls.[75,80] A large (>500 cases and >500 controls) study on co-occuring alleles revealed that of the 137

allele combinations present in >5% of women with squamous cell carcinoma of the cervix, 30 were significantly associated with an increased risk, with all but one including DQB1*0301.[81] Among the six co-expressed alleles that were associated with a decreased risk, four comprised DQB1*02.[81] The particular associations between disease and HLA class II alleles but not HLA class I alleles gain extra weight through the detection of predominant HLA class II –restricted CD4+ T-cell responses over HLA class I-restricted CD8 T-cell responses in healthy individuals and patients.[61,82,83] Furthermore a study of 49 candidate immune response and DNA repair genes revealed that a SNP in the innate immune gene IRF3 was associated with increased HPV persistence.[84] Reports on the influence of particular SNPs in the IL-10 gene are debated and those described to influence the production of the immunosuppressive cytokine TGFβ did not differ between cases and controls.[85-87] A SNP in the chemokine receptor 2 – which binds the macrophage recruiting chemokine MCP-1 – was associated with a decreased risk for cervical cancer.[86] SNPs in the promoter region of TNFγ or the receptor of IL-4 were associated with increased risk for cervical cancer.[86,88]
Other potential genetic factors involved in the progression of HPV-induced neoplasia may comprise genetic differences in the genes of the innate immune response (e.g. PRR pathways, activation of transcription factors), genes of the antigen presenting pathway, genes involved in APC activation and migration, or genes involved in T-cell migration and/or differentiation and in chemo- or cytokine production. For example, WHIM (Warts, Hypogammaglobulinemia, Infections, and Myelocathexis) syndrome is a rare congenital immunodeficiency disorder characterized by high susceptibility to HPV infection and is associated with autosomal dominant heterozygous mutations in the gene for the CXCR4 chemokine receptor.[89] WHIM is characterized by the marked reduction of circulating naïve T cells. T cells bearing this mutated chemokine receptor display an increased migratory response to CXCL12. It has been suggested that the increased migratory response results in the capture of these cells in the bone marrow [89], removing them from the periphery and as such potentially precluding their response to HPV.

Immune escape in HPV-induced disease - lessons from cervical neoplasias

Persistent viral infections reflect a failure of the host's immune system to control infection where several immune escape mechanisms of HPV are present [Box 1] (reviewed in [26,90]). While viral clearance or regression is associated with the presence of circulating CD4+ and CD8+ T-cells, viral persistence corresponds with a weak or absent early antigen specific T-cell response.[45,53,55,58,75] The systemic HPV-specific immunity to E6 and E7 is detected in approximately one third of patients with CIN or cervical cancer. The T-cell responses detected are generally not associated with the capacity to produce IFNy and can consist of Th2 cells, non-polarized T-cells, or even regulatory T-cells.[45,56,58,59,75,82,91,92] Remarkably, CD4+ and CD8+ T-cell responses in tumor and tumor draining lymph nodes in cervical cancer

are broad and aimed at both E6 and E7. When stimulated ex-vivo, they produce only low amounts of IFNy, but in the presence of APC-stimulating compounds cytokine production increases.[82,83] Interestingly, in cervical cancer patients, deep infiltration of the tumor within the normal tissue correlates with the presence of circulating HPV-specific T-cells and a better survival of patients.[75] Cumulatively, these studies suggest that the T-cells are locally not sufficiently stimulated and may even be suppressed.

Impaired antigen presentation and activation of innate immunity

Initial infection by HPV causes a cascade of viral gene expression, replication of the viral genome and enhanced cell proliferation.[26] One of the major sensors of DNA viruses is TLR9. Expression of TLR9 is either lacking or at very low levels in the undifferentiated basal cell layer of the squamous epithelia.[38] Furthermore, infection of keratinocytes with recombinant retroviruses expressing the HPV 16 E6 and E7 oncoproteins inhibits TLR9 transcription and facilitates functional loss of TLR9-regulated pathways.[93] This indicates that infected keratinocytes are not able to signal via TLR9, however, other viral PRRs might be employed.[38] Despite the presence of these other intracellular PRRs which allow infected cells to attract the immune system, the mean clearance time of HPV is 12-18 months, indicating that HPV still manages to delay or escape recognition and immune activation.[8,17] HPV does not affect the expression of different virus-sensing PRR, but genome wide expression profiling studies have demonstrated that the presence of HPV was associated with downregulation of components of the antigen presenting pathway, the inflammasome, the production of antivirals such as type I interferons, pro-inflammatory and chemotactic cytokines and activated pathogen receptors. Notably, many of the downregulated genes are found in a network that is strongly interconnected by IL-1β, a crucial cytokine to activate adaptive immunity.[38] HPV+ keratinocytes were also shown to respond less well to interferon stimulation.[94,95] This concurs with the observation that interferon-inducible genes are downregulated via inhibition of the JAK-STAT activation response pathway and downregulation of the active STAT 1 (i.e. phosphorylated or pSTAT-1), which is the primary regulator of the interferon response.[38,96]

In addition, HPV might also hamper activation of the adaptive immune system by regulating the function and migration of antigen presenting cells present in the epithelia. When viral particles are taken up by LC this does not necessarily result in an antiviral response. The structural L2 protein of HPV is able to suppress phenotypic and functional maturation of LCs and therefore can limit adequate antigen presentation to T-cells.[97,98] Furthermore, the number of LCs is reduced in HPV infected lesions compared to normal tissue.[99-101] In one of our studies the number of LCs varied extensively in cervical cancer and was related to increased migration under the influence of TNF-α.[41] The lower number of LCs is thus probably a result of lower production of chemokines and a subsequent lack of attraction of

precursor cells to replenish LC migrated out of the tissue.[42] Last but not least, the absence of pro-inflammatory signals in HPV-infected epithelia can result in inappropriately activated APCs and upon cognate interaction with T-cells they will induce T-cell tolerance.(reviewed in [102])

Alterations in Human Leukocyte Antigen (HLA) expression

Cervical cancer patients in whom circulating HPV-specific T-cells are detected display a longer survival after chemoradiotherapy than patients without a detectable T-cell response.[75] These T-cells may contribute to the antitumor response or reflect an ongoing CD8+ T-cell sustained tumor-specific immune response as they are only found in patients with HLA class I positive tumors.[103] Downregulation of HLA class I (HLA- A, -B and -C) is frequently observed in cervical neoplasia and may result in an escape of the tumor cells from cytotoxic T-cell attack.[80,104] Defects in HLA class I expression are caused by a variety of mechanisms, including loss of heterozygosity at chromosome 6p, β2-m or HLA class I mutations, defective expression and function of components of the antigen processing machinery (APM) and/or due to lack of IFNy expression.(reviewed in [105])[80,104,106] The HPV-encoded E5 and E7 proteins have also been implicated in downregulation of HLA class I.[26] Downregulation of HLA-A is associated with worse survival of patients with cancer.[107] Induction of HLA class II molecules is observed in the majority of cancers and can be mediated by cytokines such as IFNy.[108] HLA-II expression (HLA-DR, -DQ and -DP) was observed in 67% of CIN I, 58% of CIN II, 93% of CIN III and in 75% of cervical cancers.[108] In each histological category HLA-DR was most commonly expressed and HLA-DQ least commonly expressed.[108] Interestingly, analysis of CD4+ HPV 16- and 18- specific tumor infiltrating T-cells (TILs) revealed that the vast majority (>80%) of CD4+ T-cells were restricted to HLA-DQ or –DP and not to HLA-DR, suggesting that immune escape at the HLA-DQ-restricted CD4+ T-cell level may have occurred.[82] This would also agree with the protective effect of some HLA-DR/DQ combinations found to be associated with protection against HPV-induced cancer.[79] The expression of HLA-class II molecules might contribute to a successful response since activated CD4+ Th1 cells in the tumor environment enhance the recruitment, proliferation and effector function of CD8+ T-cells.[109] However, it is still unclear if the expression of HLA class II by tumor cells can also hamper the immune response and may favor tumor outgrowth as HLA class II may also induce tumor promoting signaling by rendering the CD4+ T-cells anergic after cognate interactions in the presence of immunoinhibitory cytokines and lack of co-stimulatory molecules as CD40 or by activating/inducing Tregs.[110]

Next to the classical HLA class I and II molecules, cervical tumor cells have been reported to express HLA-G. (Figure 1)[111-113] This non-classical HLA type plays an important role in tumor-driven immune escape as it inhibits the function of natural killer (NK) cells, T-lymphocytes and APCs through direct binding of inhibitory receptors immunoglobulin-like

transcripts ILT-2 and ILT-4 and the killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4).[111] The expression of HLA-G in cervical lesions is associated with progression from pre-malignant to malignant lesions and may play a role in inhibiting effective host immune responses by inducing Th2 cytokines.[111,112]

Another non-classical HLA type is HLA-E, which by engagement of the inhibitory CD94/NKG2A receptor expressed by NK and CTLs hampers the activity of these cells in the tumor.(Figure 1)[114] While NK-cells are not frequently observed, CTLs are detected in the tumors of many patients.[107,115] Notably, up to 50% of the CD8+ TILs expressed the inhibiting CD94/NKG2A receptor, whereas CD4+ TILs hardly expressed this receptor.[114] HLA-E is overexpressed in more than 80% of cervical tumors.[114]

A third non-classical HLA molecule is the MHC class I chain-related molecule A (MICA), which is expressed on normal epithelium but is weak or absent in ~60% of cervical cancer cases.(Figure 1)[107] MICA interacts with the stimulating NKG2D receptor on both CD8+ T-cells and NK-cells and enhances the effector function of these cell types.[107,116] A low expression or absence of MICA was shown to be associated with worse survival when analyzed in the context of the ratio between CD8+ T-cells and Tregs and the expression of HLA-A.[107] Downregulated expression of MICA, in addition to weak expression of HLA-A, may surpass the threshold for the infiltrating $CD8^+$ T-cells to exert their tumoricidal function.

Induction of regulatory T-cells

There is a strong correlation between the ratios of CD4+, CD8+ and tumor-infiltrating Tregs and the prognosis of HPV-induced disease.[75,107,117,118] CD4+ Tregs are shown to inhibit the proliferation and cytokine production of activated naïve CD4+ T-cells and Th1 cells and are also able to prevent the activation of CTLs by preventing the expression of the IL-2 receptor alpha (CD25) and inhibiting IL-2 production.[117] Tregs influence several other pathways to suppress the anti-tumor response, including induction of suppressive macrophages, upregulation of IL-10, induction of indoleamine 2,3-dioxygenase (IDO)-positive APCs and TGFβ production.(reviewed in [117]) In cervical dysplasia, Tregs appear to be attracted by CXCL12, a ligand of CXCR4.[119] Importantly, part of the regulatory T cell repertoire comprises HPV-specific Tregs that recognize the same antigens as HPV-specific effector cells.[117] Upon cognate interaction with the HLA class II-positive tumor cells these CD4+ Tregs become activated and can suppress other immune cells within the lesions and tumors.[58,91] Furthermore, as these antigens are also used for therapeutic vaccination strategies, vaccination may result in the expansion of HPV-specific Tregs and subsequently cause the anti-tumor response to fail.[117,118]

Inhibition of T-cell function or infiltration

Exhaustion of CD4+ and CD8+ T-cells during viral infection or malignancies has been associated with expression of the co-inhibitory molecules cytotoxic T-lymphocyte antigen-4 (CTLA-4), program death-1 (PD-1), T-cell immunoglobin mucin-3 (TIM-3) and B- and T-lymphocyte attenuator (BTLA).(Figure1)[120-123] The interaction between PD-1 receptor expressed by effector or Tregs, 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.[124-126] Approximately half of the tumor-infiltrating T-cells in cervical cancer are PD-1 positive. PD-L1, however, is only occasionally expressed by cervical cancer cells. Interestingly, patients with PD-L1 positive tumors, infiltrated with relatively high numbers of CD4+FoxP3+ Tregs, show a better survival than patients with relatively high numbers of infiltrating Tregs, but negative for PD-L1.The impairment of the PD-1 positive Tregs by the PD-L1 expressing tumor cells may potentially result in a survival benefit.[126]

The ligand for TIM-3 is Galectin 9 (Gal-9). Their interaction 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.[120,122,127] A decreased Gal-9 expression is inversely associated with malignant potential or differentiation of cervical cancer.[128] Gal-1 and Gal-3, however, have also been implied in the inhibition of T-cell responses and their expression is increased during the progression of HPV-induced neoplasia.[129,130]

Another molecule that may hamper the immune response is the cell surface glycoprotein CD200 (OX-2). This protein can be expressed by many types of human cancers.[131-134] Co-cultures of CD200-expressing, but not CD200-negative, tumor cells suppressed the production of Th1 cytokine by T-cells, the cytolytic activity of CTL and the IFNγ response of NK-cells.[131,132] In the transplantable EMT6 mouse breast cancer model, the neutralization of CD200 led to a decreased tumor growth and an increased number of cytotoxic anti-tumor immune cells in the tumor draining lymph node.[133] Tumor-cell expressed CD200 also hampers the function of tumor-associated APCs.[134,135]

Finally, T-cells can also be physically hampered to infiltrate the lesions. We found that a high expression of versican – one of the extracellular matrix components produced by stromal cells - in the stroma was associated with a low number of tumor-infiltrating T-cells and in particular a low number of CD8-positive T-cells.[136] In addition, a study of the expression of the mucosal homing receptor, α(4)β(7) surface integrin, on T-cells and its ligand mucosal addressin cell adhesion molecule-1 (Madcam-1) on vascular endothelial cells in cervical tissue revealed that the ability of α(4)β(7)(+) CD8(+) T-cells to gain access to cervical epithelium strongly depended on the expression of Madcam-1, which was absent in lesions of which the dysplastic epithelium was not infiltrated by T-cells.[137]

Microenvironment

The local microenvironment may also play a role in HPV-induced lesions. The expression of cytokines such as IL-10 and TGF-β, the increase in tumor-associated macrophages, Tregs and IDO-expressing APCs can all help to suppress local immunity. (Figure 1)[138-142] TGF-β is overexpressed in CIN and cervical cancer.[143,144] It prevents T-cell infiltration into tumors, inhibits T-cell activation and mediates Treg-induced immunosuppression.[145] The immunoregulatory enzyme IDO was found to be expressed in high grade CIN and cervical cancer, and is particularly expressed by IL-10-producing stromal myeloid cells. Diffuse expression in cervical cancer was correlated with an unfavorable outcome.[140,146,147]

Macrophages exist in many flavors ranging from a tumor-rejecting phenotype (M1 type) to the well known tumor promoting macrophages (M2 type). Monocytes recruited to lesions or tumors can differentiate towards M1 or M2 types depending on the local milieu. The M2 macrophages mediate direct effects on tumor growth, vascularisation and local immunosuppression.(reviewed in [148]) Furthermore, they produce cytokines and chemokines resulting in alteration of the phenotype and function of local DCs and the modulation of T-cell responses. The differentiation towards M2 macrophages can be the direct result of tumor cells producing prostaglandin E2 (PGE2) and IL-6. Blocking the tumor-expressed cyclooxygenase-2 (COX-2), and thereby the production of PGE2, as well as IL-6 restores the normal differentiation of monocytes to DCs.[149] Expression of COX-2 is upregulated following overexpression of E5 in cervical carcinoma cell lines.[90] The expression of COX-2 by cervical tumors is associated with a poor response to chemotherapy.[150]

Interestingly, macrophages display plasticity in their differentiation, allowing them to switch from one type to another type depending on the local milieu. Tumor infiltrated Th1 cells can stimulate a tumor rejecting environment by switching M2 tumor promoting macrophages into activated M1 tumor-rejecting macrophages via CD40–CD40 ligand interactions and the production of IFNy.[149] Another cell type reported to play a role in the suppression of immune responses are myeloid-derived suppressor cells (MDSCs). These cells are able to directly inhibit T-cell responses and promote tumor progression.[151] However, the role of both tumor associated macrophages and MDSCs in the clinical outcome of HPV-induced disease is still unclear.

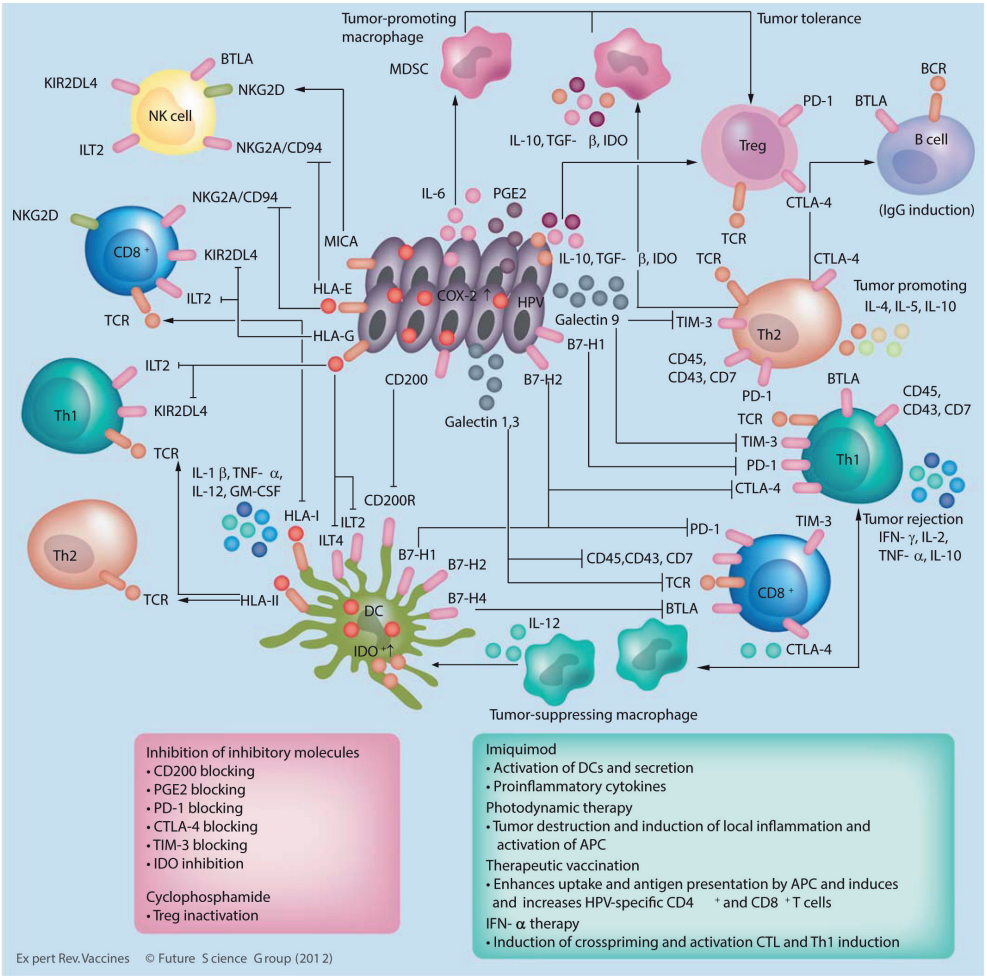

Figure 1 Immune escapes mechanisms and possible solutions

depicts all potentially involved co-inhibitory receptors, ligands and HLA expression and their role in the suppression of the immune system; TIM-3 and Galectin 9 (induction of apoptosis Th1 cells and inhibition of CTL and Th1 function), CD200R and CD200 (suppress function of Th1, CTL and NK-cells), PD-1 and B7-H1 (PD-L1) (induction of apoptosis, anergy or exhaustion of effector T-cells), CTLA-4 and B7-H1/H2 (inhibition of T-cell function), BTLA and B7-H4 (inhibition of T-cell proliferation, cytokine production, CTL function and memory cell generation), Galectin 1 and TCR, CD45, CD43, CD7, pre-BCR (induction of T-cell apoptosis, inhibition of T-cell function, Treg-mediated immune suppression and inhibition of B-cell signaling and activation), Galectin 3 and TCR, CD45, CD43, CD7 (induction of T-cell apoptosis and inhibition of T-cell function, alternative activation of macrophages), HLA-G and ILT2, ILT4 and KIR2DL4 (inhibition of T-cell function, CTL lysis, induction of tolerant APC), HLA-E and NKG2A/CD94 (inhibition of tumor cell lysis by NK-cells and CTLs), MICA and NKG2D (enhances the function of NK and CTLs). Moreover potential immunostimulating (green) and inhibition or blocking of inhibitory factors (red) to overcome some immune escape mechanisms are depicted which are of potential benefit and might improve the immunotherapy for HPV-induced neoplasia.

Immunity to HPV in vulvar intraepithelial neoplasia

VIN lesions are histologically characterized by an increased infiltration with CD4+ T-cells, macrophages but not LCs or CD8+ T-cells when compared to normal vulvar tissue.[152-155] Low-grade VIN has relatively higher numbers of CD8+ T-cells than CD4+ T-cells.[153,155] A recent study on a large series of patients and controls confirm these results with respect to the infiltration of the dermis, but also suggests that there is an increase in mature DC and plasmacytoid DC in the dermis.[152] In the epidermis, however, the number of LCs, immature DC, plasmacytoid DC and CD8+ T-cells seems to be slightly reduced.[152] The gene signature of VIN generally reflected an ongoing immune response and revealed a strong downregulation of the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), which is a negative regulator of DC maturation and function.[156] The slightly reduced number of LCs in high grade VIN can be the result of enhanced emigration via downregulation of E-cadherin and LC activation by TNF-α and IL-1β, but it may also reflect a decreased immigration of precursors due to a lower MIP-3α production by keratinocytes.[100,157] Interestingly in non-HPV-related dVIN, a number of LCs were found, suggesting that HPV induces LC activation and migration.[158] The increase of mature DCs in the dermis of VIN appears to indicate that persistent HPV infection does lead to maturation of DCs, making it most likely that disturbed immigration is the culprit in the inaccurate initiation of a strong adaptive immune response, leaving only a weak dermal influx with CD4+, CD8+ and Tregs.[152,153,156,159] Depending on the study, up to half of patients with a HPV 16-induced high grade VIN lesion display a directly detectable ex vivo HPV 16-specific IFNγ-associated type 1 T-cell response against E2, E6 and/or E7.[59,160,161] The presence of such HPV 16-specific Th1 responses is generally not associated with regression of VIN, although the clinical efficacy of treatment with imiquimod cream or electrocoagulation of the lesion is associated with the presence of such a pre-existing HPV 16-specific IFNγ-associated T-cell response.[30,31,54,59] The absence of HPV 16-specific immunity is associated with treatment failure and may contribute to the high number of recurrences after treatment.[29-31,162] The critical role of a strong and broad systemic and local CD4+ and CD8+ HPV 16-specific proinflammatory T-cell response in order to clear usual VIN is demonstrated by therapeutic vaccination studies.[29,31,60,161,163-166] HPV 16 L1-specific serological responses are detected in the great majority of VIN patients but are not related to clinical outcome and is associated with a risk of developing VIN.[59,162,167] NK cells are sporadically found in the epidermis but were found to be more than doubled in numbers in the dermis of VIN lesions compared with healthy women[152] and may reflect co-infiltration with dermal effector T cells and Tregs. Furthermore, in VIN, the intensity of COX-2 expression analyzed by immunohistochemistry is not correlated with the degree of vulvar dysplasia.[168]

Similar to the other HPV-induced anogenital diseases, people carrying the HLA-DRB1*13 and -DQB1*05 alleles are associated with a decreased risk of HPV 16-induced high grade VIN or vulvar cancer, whereas HLA-DQB1*03 is strongly associated with an increased risk.[169] Downregulation of HLA class I was found in nine out of 11 vulvar carcinomas (82% total loss), irrespective if they were HPV-induced or not.[155] In these 11 carcinomas HLA-class II expression was not upregulated.[155] In VIN, 19% total loss of HLA-class I was observed both in dVIN and uVIN. In addition, in 21% of usual VIN biopsies HLA-class I was found to be downregulated.[155] Whilst there are many mechanisms leading to changes in HLA expression[105], which part is still reversible via IFNγ remains to be established, particularly since the presence of an HPV-specific IFNγ-associated T-cell response is correlated with regression of lesions.

In vulvar neoplasia both Gal-1 and Gal-3 were shown to be upregulated from normal vulvar tissue to high grade VIN and vulvar carcinoma.[170] The expression of Gal-1 on macrophages adjacent to the neoplastic cells became stronger with the increase in disease severity, whereas the neoplastic cells stained negative or weak for Gal-1.[129,170] Gal-3 staining was observed mostly in the epithelial cells but was also found in endothelial cells and macrophages. The vast majority of VIN cases did not express Gal-3. However, in approximately 60% of the vulvar carcinomas, Gal-3 expression by the cancer cells was moderate or strong.[171] The potential role of these and the other co-inhibitory factors discussed above in the T-cell response to HPV-induced VIN need to be determined.

Immunotherapy and potential causes of failure in VIN

A successful immunotherapeutic approach probably requires resetting many parameters in the immune response to HPV. Therefore, immunotherapy for VIN must be aimed at adequate priming of T-cells, altering the balance between effectors and Tregs, increasing the homing of T-cells to the lesion, preventing exhaustion of the immune response, and overcoming inhibition by creating an immune stimulatory environment that allows the immune system to work.[172] At present, a number of immunotherapeutic approaches have been tested with varying degrees of clinical success. Although some of these approaches showed some clinical efficacy in patients with VIN, a substantial number of cases failed to display a clinical response to immunotherapy. The potential causes of which will be discussed below.

Topical immunotherapy

In the early 1990s, 21 patients were treated with topical IFNγ with or without nonoxynol-9. Nine patients displayed a complete response for at least 1 year and overall 67% of the patients showed an objective clinical response. However, due to serious local side effects and high costs this therapy is no longer pursued.[173,174] More recent data showed that IFNα plays an important role in the activation of long-lasting anti-tumor responses.[175] IFNα drives

the generation of IFNγ-producing Th-cells and CTL as well as promoting the proliferation and survival of T-cells.[176] The use of pegylated IFNα may overcome the side effects previously observed in IFNα therapy of VIN patients because it differs in pharmacokinetic and chemical properties and is associated with fewer side effects compared to the topically used one.[177]

Imiquimod (Aldara®) is a topically applied immune response modifier that acts by binding to TLR7 resulting in activation of NF-kB, which is followed by secretion of multiple pro-inflammatory cytokines such as TNF-α, type 1 IFNs, IL-12 and activation of DCs.[178,179] Topical imiquimod has been used to treat HPV-induced high grade VIN lesions and resulted in viral clearance, normalization of immune cell infiltrate and clinical responses even in long-term follow up.[28,32,33,180] Complete regressions were observed in 26–100% of patients whereas 0–60% displayed partial regression and 0–37% experienced recurrence.[180] In two randomized controlled trials with imiquimod as treatment of high grade VIN, the first trial demonstrated complete regression in 81% of the 21 patients and partial regression in 10%, whereas the other trial reported a complete regression in 35% and partial response in 46% of the 26 patients.[28,32] Treatment in general is well tolerated, however local side effects of inflammation and burning are common, but can safely and successfully be treated with Non-Steroidal Anti-Inflammatory Drugs (NSAIDs).[28,32,181] Regression of the lesions in the last study was associated with a pre-existing IFNγ-associated HPV 16-specific CD4+ T cell response and afterwards with normalization of the lesional immune cell infiltrate.[59,159] Imiquimod did not induce or enhance the HPV 16-specific CD4+ T-cell response.[59] Non-responsiveness corresponded to the local attraction of macrophages and the presence of Tregs.[31,159] Notably, HPV 16-specific Tregs comprise both FoxP3+ and FoxP3- suppressor cells and are characterized by the production of both IFNγ and IL-10.[91] It can be envisaged that they also play a role in VIN lesions as they are found in high grade premalignant lesions of the cervix.[58]

Photodynamic therapy (PDT) in combination with topically applied 5-aminolevulinic acid (ALA) is a relatively new treatment regimen for VIN that exploits the interaction between a tumor-localizing photosensitizer and non-thermal light to induce oxidation reactions, which lead to tissue necrosis.[182] ALA-based PDT is particularly attractive as this drug is activated in rapidly growing cells, thereby reducing damage to the normal surrounding tissue.[182] PDT results in direct tumor destruction as well as induction of local inflammation resulting in the activation of APCs, recruitment of effector cells and subsequently the activation of tumor-specific immunity and development of immune memory.[183,184] PDT can also have an immunosuppressive effect, but this is prevented by reducing the rate of light delivery.[185] Clinical responses of VIN to PDT vary widely ranging from 20 to 60% of complete histological responses and in 52–89% of the patients it resulted in symptom relief.(reviewed in [186]) Curative responses were more frequently observed in unifocal lower grade VIN and

non HPV-associated VIN as well as in pigmented and hyperkeratinic VIN lesions.[155,187] Moreover, clinical responders retained the expression of HLA-class I and displayed a treatment-associated increase in the numbers of infiltrating CD8+ lymphocytes. In contrast, non-responders showed loss of HLA-class I and low numbers of infiltrated immune cells. [155,187]

Advantages of PDT are a short healing time, minimal tissue destruction and preservation of the normal anatomy of the vulva. However, recurrence rates are high (48.7%) and do not differ significantly from conservative treatments with CO2 laser and surgical excision. [12] In an attempt to increase the immune infiltration and response to PDT of HPV-induced VIN, a combination therapy of imiquimod and subsequent photodynamic therapy was given. The overall response rate was 65%, with 20% complete responders and 40% partial responders after 1 year.[30] Indeed, imiquimod treatment resulted in an increased CD8+ T-cell infiltration in the group of treated patients, but no differences were found between non-responders and responders within this treated group. Non-responders demonstrated relatively stronger infiltration with FoxP3+ T-cells after imiquimod. Clinical responders displayed a stronger HPV-specific proliferative T-cell response.[30] Together, these studies indicate that topical immunotherapy, particularly imiquimod, changes the microenvironment to allow more immune cells to infiltrate into the lesions. When these T-cells display the correct phenotype, as potentially found in patients with circulating HPV-specific T-cells, this infiltration may result in clinical responses. By contrast, when the immune cells display an immune suppressive phenotype (Tregs, M2 macrophages) the patients will not successfully react to the therapy.

Systemic immunotherapy by vaccination

Therapeutic vaccines aim to reinforce the HPV-specific IFNγ-associated CD4+ and CD8+ T-cell responses. As previously reviewed, different types of therapeutic vaccines have been developed and tested in Phase I/II clinical trials in an attempt to eliminate HPV 16-associated disease. The types of vaccines comprise recombinant viral vectors, peptides, fusion proteins, DNA, antigen-pulsed DCs and virus-like particles.(reviewed in [188]) Although some vaccines showed high immunogenicity, vaccination has led to limited clinical successes in HPV induced diseases. Part of it can be explained through the failure of some vaccines to induce a strong and broad HPV 16-specific CD4+ and CD8+ T-cell response.(reviewed in [188])

Different vaccine formulations have been tested in patients with HPV-induced VIN. In general, these vaccines were shown to be safe and immunogenic in most cases. A live recombinant HPV vaccine expressing the HPV 16 and HPV 18 E6 and E7 genes (TA-HPV) was tested in 12 women with high grade VIN or VAIN of up to 15-years duration in a single dose injection. [161] A total of 42% of the patients showed at least a 50% reduction in total lesion size and in one patient a complete regression of the lesion was achieved.[161] The vaccine induced

an increased IFNγ-associated HPV-specific T-cell response in six patients, who showed a concomitant clinical response.[161] However, in four women no vaccine-induced T-cell response was observed while two of them showed a >50% reduction in lesions size.[161] This vaccine was also tested in 18 women diagnosed with VIN that had persisted for 6-months to 17-years. Eight patients showed an increase in the HPV E7-specific IFNγ response, of which four displayed a weak response. One patient showed a complete clinical response and seven others a partial response. There was no obvious correlation with the vaccine-induced response, probably because only the response to two well defined HLA-A*0201 restricted CTL epitopes was measured. E6/E7-specific proliferative responses were measured in 50% of the patients.[164] Interestingly, a comparison of the local immune infiltrate revealed that the lesions of the group of clinical responders were, on average, highly infiltrated with DC/LCs, CD4+ and CD8+ T-cells before vaccination. There was no difference in the number of CD68+ macrophages between the two groups. Notably, the numbers of VIN-infiltrating CD4+ and CD8+ T-cells did increase in the group of non-responders after vaccination, albeit not to the level of the clinical responders.[164] This indicates that the capacity of the immune cells to infiltrate the lesion represents another hurdle to be overcome.

Another vaccine formulation consisting of HPV 16 L2E6E7 fusion protein (TA-CIN), which was given to ten women diagnosed with high grade VIN as three booster vaccinations after they were primed with TA-HPV 7–15 months earlier.[165] All patients displayed a proliferative response to the L2E6E7 fusion protein, but it is not clear how often this response was made against the L2 component. IFNγ-associated CD8+ T-cell responses to two of the HLA-*0201 restricted E7 epitopes were detected in three patients. One patient showed a complete response and one a partial response (>50% reduction in lesion size). There was no correlation found between the outcome of the immune assays and clinical reactivity.[165,166] Preclinical studies on heterologous prime-boost immunizations with TA-CIN and TA-HPV showed that the best vaccination protocol would consist of priming with TA-CIN to focus the immune response toward the oncoproteins, and boosting with TA-HPV to increase the magnitude of the E6/E7-specific response. Therefore, a group of 27 patients with HPV 16+ VIN 3 and two patients with VAIN 3 was vaccinated with three doses of TA-CIN at four week intervals followed by a single boost of TA-HPV. This resulted in one complete response and five partial responses.[166,189] Analysis of the HPV 16 E6/E7-specific IFNγ-associated T-cell response using pools of overlapping peptides of E6 and E7 was performed in 25 patients, three of which showed a pre-existing response to E6. In nine patients, the vaccine-induced IFNγ-associated T-cell response was detected after vaccination, including two of the patients with a pre-existing immune response. The responses were mainly focused at the E6 protein and not at E7. Two patients showed a strong IFNγ-associated T-cell response. Of the six clinical responders, five patients showed an E6-specific IFNγ response, including the complete responder.[166] Notably, the combination of TA-CIN and TA-HPV – although

a strong combination in mouse models - resulted in no advantage over a single TA-HPV vaccination in patients with VIN.[166,189]

Peptide vaccines are attractive because they are well tolerated in humans, relatively inexpensive to produce and easy to design. A vaccine consisting of synthetic long peptides (SLP), spanning the complete amino acid sequence of the two oncogenic proteins E6 and E7 of HPV 16, was safe and highly immunogenic in patients with cervical cancer as reflected by the strong IFNγ-associated HPV 16-specific CD4+ and CD8+ T-cell responses detected. [190,191] In a Phase II trial, 20 patients with high grade HPV 16+ VIN were vaccinated three to four times at 3 week intervals.[29] After 12 months follow-up, a clinical response rate of 79% and a complete and durable regression of the lesion in 47% of the patients was reported. Patients with a good clinical response displayed significantly smaller lesions at study entry than those who did not.[29] Characteristically, patients with a smaller VIN3 lesion displayed a strong and broad IFNγ-associated HPV-specific CD4+ T-cell response to HPV 16 E6 and E7. There was no difference between patients with small or larger lesions in the immune response to recall antigens. The patients with a smaller lesion displayed a distinct peak in the amount of cytokines produced by peripheral blood mononuclear cell (PBMCs) isolated after the first vaccination, suggesting that HPV 16-specific immune responsiveness is already predetermined. By contrast, in patients with larger lesions, higher frequencies of vaccine-enhanced HPV 16-specific Tregs were observed.[29,60] At present in our institute, a Phase II randomized trial in patients with HPV 16 positive high grade VIN is almost completed in which imiquimod is applied to site where the HPV 16 E6/E7-SLP vaccine is injected in an attempt to improve the Th1 polarization of the responding T-cells and as such clinical responses. In a peptide-based vaccine in patients with melanoma, imiquimod appeared to enhance the immunological response to the vaccine.[192] As a response to the observation that non-responders to PDT exhibited a loss of HLA-class I, a pilot study was performed on the available pretreatment biopsies of the HPV 16-SLP vaccinated patients. This revealed that (partial) loss of HLA-class I in the VIN lesions could be observed before vaccination across non and clinical responders, indicating that (partial) loss of HLA does not always prevent successful outcome of vaccination.[unpublished results] The expression of HLA class I may potentially be restored by the presence of IFNγ produced by HPV-specific CD4+ T-cells that recognize their epitope in HLA-class II, with as a result that the recruited CTL once again can exert their cytotoxic function.[109] Alternatively, CD4+ T-cells have a direct antiproliferative effect on HLA class II positive cells of the lesion.

Combined local and systemic immunotherapy

Clearly, there are a number of immunotherapeutic strategies that are promising for the treatment of VIN, but are not able to induce complete regressions of the lesions in every single patient. Based on the finding that clinical responsiveness to vaccination is associated

with the extent of the pre-treatment immune infiltrate in high grade VIN[164] and that this infiltrate can be enhanced by the use of imiquimod[30], a vaccine trial was designed in which imiquimod treatment for 8 weeks was followed by three intramuscular doses of TA-CIN at 4-week intervals.[31] A total of 19 women with (1–20 years of) high grade VIN were treated. After treatment with imiquimod, six patients showed a complete regression and this increased to 11 women after vaccination with TA-CIN. After one year of follow-up, 12 of the 19 treated women displayed a complete regression.[31] The group of clinical responders showed a stronger E6- and E7-specific proliferative response than non-responders. Imiquimod treatment was expected to raise the numbers of lesion infiltrating immune cells. Indeed a small but significant increase in the numbers of CD8+ T-cells was detected. After vaccination, the group of responders showed an increased CD4+ and CD8+ T-cell infiltration. By contrast, the group of non-responders showed an increase in the number of lesion-infiltrated Tregs.[31] In comparison to their previous study where imiquimod was followed by PDT, not only the number clinical responders was much higher, but also the numbers of infiltrating CD4+ and CD8+ T-cells in the lesions did not return to pre-imiquimod levels as was seen with PDT[30], suggesting that the vaccine-induced HPV 16-specific T-cell response mediated this effect. However, similarly to what was observed after HPV 16-SLP vaccination, TA-CIN vaccination resulted in the undesirable side effect of enhanced the number of Tregs in the group of non-responders.[31,60]

Conclusion and Future

Overall, one can conclude that clinical complete regression of HPV-induced disease can be obtained if the numbers of HPV-specific CD4+ and CD8+ T-cells are strongly enhanced and the lesions are (preconditioned to) allow immune cell infiltration. Clearly, a number of pre-existing conditions (e.g. lesion size, presence of Tregs, lack of immune infiltration, lack of HPV-specific IFNγ-producing T-cells, number of infiltrated macrophages, lack of HLA class I expression) may determine the patient's responsiveness to immunotherapy. Other factors that may influence responsiveness to therapy, such as the type of infiltrating macrophages (M1 or M2), the presence of MDSCs, the expression of Madcam or the expression of inhibitory molecules, which are all known to influence the immune response in other HPV-induced or chronic viral diseases, still need to be addressed. The consistent observation that non-responders display enhanced numbers of Tregs after therapy suggests that new modalities should be sought that alter the balance between Tregs and IFNγ-producing effectors in favor of the latter. Tregs are known to suppress the induction of the IFNγ-producing Th1 cells that are required to mobilize and sustain CD8+ T-cells to the site of disease.[109,193,194]

Expert commentary

VIN is the first HPV-induced disease in which real immunotherapeutic clinical successes have been achieved. In comparison to CIN and cervical cancer, VIN is, however, less well studied with respect to its interaction with the immune system. Most studies on the immune response to HPV in uVIN have been performed in clinical trials. On the one hand, this allows immune parameters to be studied in the context of therapy-induced outcome and as such may reveal parameters associated with success or failure. On the other hand, such studies are generally focused on the mechanism of action of the therapy and therefore do not consider other immune factors. Furthermore, most studies are performed in small cohorts of patients which limits the value of findings or significantly obscures potentially important findings. Although it is acceptable to translate findings from other HPV-induced diseases to the study of VIN, they do need to be confirmed preferably in larger cohorts with known clinical follow-up. Gaps in the knowledge of the interaction of HPV and the immune system in VIN include the absence or limited studies on (non-classical) HLA expression, the presence of co-inhibitory molecules or the presence of local inhibitory microenvironmental factors as macrophages and cytokines. Considering that VIN does respond to immunotherapy, in-depth studies should be performed to fully understand why it works, as well as to understand what we need to circumvent in patients who would otherwise not respond for immunotherapy of HPV-induced tumors to be successful. Based on what is already known, we think that HPV-induced VIN is an immunologically active disease as reflected by marked infiltration with T-cells and the presence of IFNγ-associated T-cell responses, when compared, for instance, to HPV-induced CIN. Importantly, while evidence accumulates that HPV-specific T-cells play a role in all HPV-induced diseases, the results of the vaccine trials definitely show that HPV-specific T-cells play a role in the control and regression of VIN. Moreover, these trials show that even high grade HPV-induced lesions can undergo immune-driven regression. However, when HLA expression is lost, infiltration with Tregs and macrophages, or the *per* individual differences in immune infiltration is considered, VIN is similar to the other HPV-induced high grade lesions or cancers. It is therefore likely that the development of new treatment options of this premalignant lesion will follow the same route as that for cancer. Here, one must consider applying immunotherapeutic vaccines in the adjuvant setting but may also include the use of low dose chemotherapy to obtain certain immunological effects such as Treg depletion. For instance, a single dose of cyclophosphamide improved IFNγ-associated T-cell responsiveness to vaccination in ovarian cancer.[195] In view of what has been found already, it is highly likely that a combination of immunotherapeutic strategies is required to increase success rates in the treatment of VIN.

Five-year view

At present the successes of different (combined) immunotherapies in the treatment of VIN are encouraging and unprecedented. However, non- or partial treatment responders are also being reported and this will spark studies that focus on unraveling the mechanisms underlying the differences in clinical responsiveness. Interestingly, there are several roads that lead to clinical success. These – in our opinion – complementary immune strategies all target different aspects of immune failure. We expect that combinations of these strategies will be tested. To the end, this will lead to either patient selection in cases where there is no strategy to overcome an immunological problem or to a number of combinations of immunotherapeutic strategies that together may solve the problems. One may consider, for instance, blocking the co-inhibitory molecules by antibodies, depletion of Tregs before therapy, depletion or re-differentiation of macrophages, increasing T-cell homing by the induction of local inflammation and the use of IFNα to polarize Th1/CTL responses. Combination of imiquimod, vaccines and IFNα are the most likely to be tested within the near future.

Key-issues

- Usual type VIN is a chronic premalignant disease caused by a persistent oncogenic HPV 16 infection in 90% of cases.
- Successful treatment of HPV-induced VIN is associated with an enhanced and broad HPV 16-specific CD4+ and CD8+ T-cell response against the oncoproteins E6 and E7.
- Several types of immunotherapeutic approaches have met clinical success in high grade VIN although a notable number of patients fail to respond to these immunotherapeutic strategies.
- Knowledge of the interaction between VIN and the immune system is limited.
- Immune escape mechanisms that play a role in VIN are loss of HLA, immune infiltration with Tregs and macrophages, or the lack of infiltrating CTLs.
- Studies on the role of inhibitory molecules on T-cell function and the reversibility of HLA loss in VIN are needed.
- Combinations of immunotherapies targeting different aspects of the failing immune response may overcome immune escape and enhance clinical response rates.

Box 1 Potential causes of immunotherapy failure and how to correct this (Figure 1)

Lack of a robust CD4+ and CD8+ HPV-specific T cell response.
The accumulated data show that a strong and broad IFNγ-associated HPV-specific T-cell response is required for complete regression of the VIN lesion. Although some patients spontaneously develop a respectable HPV-specific immune response, many do not. Immunotherapeutic strategies that do not comprise vaccination may fail in patients either by lacking HPV-specific immunity or by developing a dominant Th2 response.

- *Lack of HPV-specific immunity*: The immune system should come into contact with sufficient amounts of antigen for sustained periods to become activated. The first choice would be to vaccinate patients with a highly immunogenic vaccine that induces both CD4+ and CD8+ T-cell responses. Alternatively, one could use lesion destructive therapies that are known to result in the activation of T-cells such as PDT or cryoablation.
- *Wrong polarization of HPV-specific immunity*: Stimulation of the immune system with E6 and E7 antigens (delivered to the APC via natural mechanisms, vaccines or destructive therapies) to induce a strong Th1/CTL polarization requires optimal activation of APC. TLR 3 and 9 agonists are known to appropriately stimulate human APC and could be injected either locally or at the site of vaccination. Alternatively, one could use pegylated IFNγ as this is known to prevent Th2 responses and to drive Th1/CTL responses.

Inhibition of T-cell function and infiltration.
As a group, VIN lesions are well infiltrated with CD4+ and CD8+ T-cells, however, this varies a great deal between individuals. Despite strong infiltration with T-cells, they are incapable of inducing the spontaneous regression of VIN lesions. Immunotherapeutic strategies aiming to induce HPV-specific immunity have a better response rate in patients displaying considerable immune infiltration before treatment, except when there are high numbers of regulatory T-cells present.

- *Regulatory T-cells*: Non responsiveness to therapy is associated with the presence as well as enhancement of regulatory T cell numbers. Part of the regulatory T-cell population is HPV-specific. In the immunotherapeutic strategies that aim to induce HPV-specific immunity, an increase in circulating and local regulatory T-cells is observed in clinically non-responding patients. Preferably, modalities to deplete or disarm regulatory T-cells should be included in the immunotherapeutic strategy. A number of different methods have been tried without much success. So far, low dose cyclophosphamide treatment has produced consistent results, suggesting that low dose chemotherapy should be considered as a treatment option. Since it is not the number of regulatory T-cells *per se* but the balance between regulatory T-cells and effectors that is important one could try to use adjuvants that alter the balance between these two populations in favor of the effector cells.
- *Expression of co-inhibitory molecules:* Overall there is little data on the expression of co-inhibitory molecules by T-cells, immune cells in the microenvironment or the epithelial cells in patients with VIN. Clearly, the increase in severity of VIN lesions is associated with an increased expression of galectin-1 and galectin-3 in VIN. These galectins are known to disrupt T-cell function and to induce apoptosis in melanoma. Similarly, the interaction between T-cell-expressed TIM3 and galectin-9 disrupts T-cell function. The use of galectin inhibitors or antibodies to TIM-3 may alleviate inhibition. The role of other inhibitory molecules has not been studied in VIN but extrapolation of what is known from other HPV-induced diseases and other types of cancers, suggest that PD1 and CTLA-4 may play a role. For the latter molecule a clinically effective blocking antibody has been approved for melanoma, whereas for PD-1, several clinical grade antibodies are being developed. These might be added to the immunotherapeutic strategy. Potentially, CD200/CD200R interaction may also play a role. An antibody to CD200 has been tested in the treatment of B-cell leukemia (ClinicalTrials.gov Identifier: NCT00648739)

- *Inhibition T-cell infiltration:* A lack of Madcam-1 expression on the vasculature of CIN lesions was associated with a failure of T-cells to infiltrate the lesions and this may also explain why in a number of patients VIN lesions are not infiltrated by T-cells or not reactive to therapy. Madcam-1 is upregulated by TNFα, which may explain why the use of topical imiquimod can enhance immune infiltration of VIN lesions.

Microenvironmental factors.

- *Lesion associated macrophages:* VIN lesions display an increased infiltration with macrophages and non-responsiveness to imiquimod treatment corresponded with a local attraction of higher numbers of macrophages, suggesting that the macrophages detected display a tumor-promoting M2 profile and not the tumor-rejecting M1 profile, but this is yet to be confirmed. In HPV-induced cervical cancer, IL-10 producing M2 macrophages can be induced by tumor produced PGE2 and IL-6. COX-2 is variably expressed in VIN[168], but the expression of IL-6 is unknown. If M2 macrophages are involved, treatment could consist of IL-6R blocking antibodies (*e.g.* Tocilizumab) as used in rheumatoid arthritis and COX inhibitors. Alternatively, it has been shown that the interaction between Th1 cells and M2 macrophages switch the latter to activated IL-12 producing M1 macrophages. This suggests that if an immunotherapeutic approach results in enough lesion infiltrating Th1 cells, there might even be a benefit from the macrophages as they help to change the microenvironment to become more favorable.
- *IDO*: The expression of IDO is found in high-grade CIN and cervical cancer, where it is associated with clinical outcome. The IDO inhibitor 1-methyltrypthophan may be utilized if IDO plays a role in VIN.
- *MDSC*: myeloid-derived suppressor cells in many cancers can suppress the infiltrated effector T- cells by various mechanisms, although their exact role remains to be determined.

The expression of classical and non-classical HLA molecules.

- *Loss of HLA-class I (-A,-B,-C):* Downregulation of HLA-class I molecules may hamper the efficacy of HPV 16-specific CD8+ T-cells to exert their function. If this downregulation is irreversible meaning that there are genetic alterations which cannot be restored one may deselect such patients for immunotherapy. If the downregulation is reversible, meaning that it can be restored by IFN, then an increased infiltration of the lesion with Th1 cells should suffice to restore HLA expression. Alternatively, a local injection with pegylated IFNα or IFNγ during the treatment may help to promote HLA expression.
- *HLA-G:* In CIN lesions the expression of this molecule is associated with progression and the induction of Th2 responses. No intervention options are yet available.
- *HLA-E:* This molecule is expressed by the majority of cervical cancer where it can bind to the inhibiting CD94/NKG2A receptor expressed by up to 50% of the tumor-infiltrating CD8+ T-cells. No blocking antibodies have been developed to prevent this interaction to occur.
- *MICA:* This molecule binds to the co-stimulatory molecule NKG2D expressed by CD8+ T-cells. Downregulation of this molecule by cervical cancer cells is associated with lower patient survival. MICA is known to be upregulated by TNFα, suggesting that a local pro-inflammatory reaction may rescue MICA expression, but also by gamma-radiation.[196]

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