Towards a tailored therapeutic approach for vulvar cancer patients

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THE IMMUNE MICROENVIRONMENT IN VULVAR (PRE)CANCER: REVIEW OF LITERATURE AND IMPLICATIONS FOR IMMUNOTHERAPY

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ABSTRACT

Introduction

Vulvar squamous cell carcinoma (VSCC) develops via two different pathways: *TP53* mutations in a background of lichen sclerosus or a persistent infection with high-risk human papilloma virus (HPV). The latter group of tumor responds better to treatment than the non-virally induced VSCC. This may be explained by a difference in the tumor immune microenvironment (TME).

Areas covered

This review summarizes literature on TME of VSCC and its precursors, and extrapolates this to foster the development of new therapeutic strategies.

Expert opinion

Both types of VSCC and their precursors are infiltrated with variable numbers of M2 macrophages, regulatory T cells and $CD8^+$ T cells, indicating that they express targetable tumor antigens. Type 1 T cell immunity in precursor lesions is associated with fewer recurrences and better clinical responses to immunotherapy. Escape of these lesions and progression towards VSCC is associated with the downregulation of HLA class I, increased expression of co-inhibitory molecules, infiltration with immunosuppressive cells and the local production of immunosuppressive enzymes and cytokines. More in-depth studies of the VSCC TME are required to fully comprehend the impact of the immune system on VSCC, and subsequently to identify patients who will benefit from immunotherapeutic strategies.

INTRODUCTION

Vulvar cancer is a rare disease, representing 3-5% of all gynecological malignancies, with an increasing annual incidence of 1-2 per 100.000 women.[1, 2] Around 90% of all vulvar tumors are vulvar squamous cell carcinomas (VSCC).[3] Two independent pathways have been identified in the pathogenesis of VSCC (figure 1). Classical development of VSCC is related to *TP53* mutations (HPVneg VSCC) accounting around 80% of all VSCC.[4] Persistent infection with high risk human papillomavirus (hrHPV) causes the other 20% (HPVpos VSCC).[5, 6] The overall incidence of VSCC is rising, likely due to higher life expectancy and the overall increase in HPV infections.[7] The standard treatment for VSCC consists of surgery and/or radiotherapy and is associated with high morbidity rates and impaired quality of life.[8]

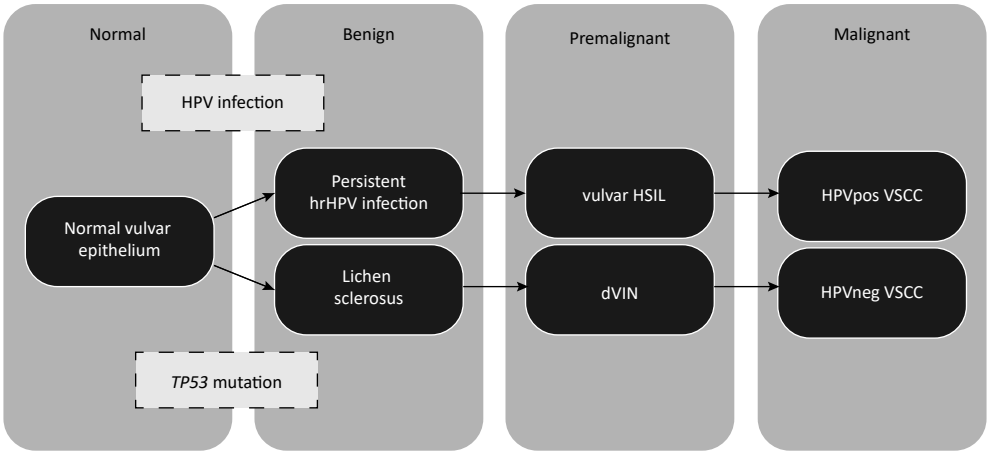

Figure 1. Overview of the multistage evolution of VSCC, depicting its two distinctive pathways. The HPV-associated pathway starts with an HPV infection, of which the lifetime risk for women is 80%. 20% of these infections persist, of which half causes vulvar high-grade squamous intraepithelial lesion (vHSIL). A small percentage of vHSIL develops into HPV-associated VSCC. The HPV-independent pathway is associated with lichen sclerosus, which can develop into dVIN, commonly after *TP53* mutation. dVIN is considered a direct precursor of HPV-independent VSCC, which accounts for 80% of all VSCC cases.

The precursor of HPVneg VSCC is vulvar intraepithelial neoplasia of the differentiated type (dVIN) and often arises in a background of lichen sclerosus (LS).[5, 9] It is suggested that dVIN, rather than LS, is the direct precursor of HPVneg VSCC.[10] HPVneg VSCC is associated with *TP53* mutations, which leads to abnormal expression of p53 protein.[5, 11] Somatic mutations in *TP53* lead to an uncontrolled cell cycle and chromosomal instability, resulting in tumor formation.[12] In HPVpos VSCC ~75% of the lesions are caused by HPV type 16, one of the most dominantly present oncogenic high-risk HPV types (hrHPV).[6] HPVpos VSCC's precursor lesion is vulvar high grade squamous intra-epithelial lesions (vHSIL), formerly referred to as vulvar intraepithelial neoplasia of the usual type (uVIN). Cells infected by hrHPV express the viral oncogenes E6 and E7, which disturb the regulation of the cell cycle and promote malignant conversion. The lifetime risk of acquiring an hrHPV infection is approximately

80% for sexually active individuals. About 80% of the infections are cleared by the immune system within 18 months.[13] Less than 10% of the infections persist and may cause vHSIL, 6.5% of which subsequently progresses towards VSCC.[9, 14] Several retrospective studies suggest that patients with HPVpos VSCC display a better progression-free survival when compared to patients with HPVneg VSCC.[11, 15-17] Potentially, this difference is due to a more pronounced type 1 immune response as seen in various other malignancies.[18]

The adaptive cellular immune system is known to play a role in the protection against HPV-induced lesions. The group of immunosuppressed individuals (e.g. HIV, transplant patients and patients with leukemia) shows a higher incidence in persistent HPV infections and HPV-related malignancies.[19-21] In addition, complete regression of HPV-induced lesions is associated with a strong influx of $CD4^+$ and $CD8^+$ T cells.[22] Furthermore, healthy individuals display *ex-vivo* measurable frequencies of HPV-specific interferon producing T cells[23], which are largely lacking in patients with HPV-induced cancer.[24]

Surprisingly though, the immune response in VSCC has not been studied thoroughly. Potential differences based on the distinct etiological pathways leading to HPVpos VSCC and HPVneg VSCC have largely been ignored. This could be key to our understanding of the clinical behavior of VSCC. The expressed viral onco-proteins can function as tumor-specific antigens (TSAs) in HPVpos VSCC, fueling the induction of a strong local type 1 T cell response and potentially leading to the better clinical outcome of HPVpos VSCC when compared to HPVneg VSCC.[11, 17] Probably, the development of VSCC parallels that of oropharyngeal squamous cell carcinoma (OPSCC), which also develops via a non-viral or an hrHPV-driven pathway.[25] In this tumor type, the presence of HPV was found to be a strong independent determinant of progression free survival[26, 27] and associated with a dense infiltration of activated $CD4^+$ and $CD8^+$ T cells.[26, 28] Especially the intratumoral presence of type 1 cytokine producing HPV16-specific T cells constituted a type 1 immune contexture and improved clinical outcome. The absence of intratumoral HPV-specific T cells led to worse clinical outcome, indistinguishable from that of most HPVneg OPSCC.[29] The recognition that HPVpos OPSCC have a better clinical outcome led to new trials of de-intensified chemo (radio) therapy schedules, testing of novel therapeutics, including immunotherapeutic compounds in OPSCC.[30-33] Hence, HPVpos VSCC and HPVneg VSCC are different entities and they should be studied separately. Here, we summarize studies on the immune response as measured in the local tumor microenvironment (TME) in VSCC, and correlate this information to what is known about the precursors of VSCC and other HPV-induced malignancies. In addition, we will extrapolate this information to briefly delineate future therapeutic options for VSCC.

INNATE IMMUNE RESPONSES IN VULVAR LESIONS

The role of the innate immune system is ambiguous, since it may promote tumor growth as well as prevent tumor progression.[34, 35] For instance, the role of tumor-associated myeloid cells is to foster tumor progression. Myeloid derived suppressor cells and type 2 macrophages can promote initiation and metastasis of tumor cells, and inhibit tumor-specific T cell responses.[36] On the other hand, abundantly present M1 macrophages in HPV-driven cervical cancer are an independent prognostic factor for good survival[37], and interferon-γ (IFN-ɣ)-activated macrophages are important for tumor growth control in an HPV-tumor animal model.[38, 39] First, the role of macrophages will be discussed and then the focus is on professional antigen presenting cells (APCs), as these are crucial for the induction of adaptive immune responses.

In one study, myeloid cell infiltration between HPV16 driven primary vHSIL (*n*=43), 20 recurrent vHSIL, 21 VSCC and 26 normal vulvar tissues was compared.[40] Progression was associated with an increase in the numbers of $CD14^+$ cells. Both in healthy controls and $HPV16^+$ vHSIL the $CD14^+CD33^-CD163^+$ mature (activated) type 2 macrophages were more abundantly present than the M1 macrophages. Importantly, a higher number of both intraepithelial and stromal mature $CD14^+$ macrophages was positively correlated to the number of intraepithelial regulatory T cells (Tregs, defined as $CD4^+Foxp3^+$, $CD3^+PD\text{-}1^+Foxp3^+$), negatively correlated to the number of activated CD8 T cells as well as with a decreased recurrence free survival time in vHSIL.[41] The M2 macrophages are immune suppressive as reflected by their production of IL-10 and TGF-β. Potentially, M2 macrophages induce local Treg polarization.[42] High type 2 macrophage counts are associated with poor outcome in a diversity of tumors[43-45], suggesting that they may have a similar negative impact in VSCC. Indeed a large study of 100 VSCC confirmed that these tumors were infiltrated with variable numbers of $CD68^+$ macrophages and T cells.[46] It also showed that increased immune infiltration of the tumors was associated with the expression of PD-L1 in 20% of the HPVneg VSCC although this was not observed in the HPVpos VSCC.[46] The expression of PD-L1 was also found in a mouse tumor model[38] and may reflect an ongoing antitumor response in those HPVneg VSCC, suggesting that also HPVneg VSCC can express antigens that are available for the immune system to react to. Unfortunately, no TME studies have been performed in dVIN, albeit that increased numbers of T cells and $CD68^+$ macrophages were found in LS.[47]

The professional APCs such as Langerhans cells (LCs) and dendritic cells (DCs) connect the innate and adaptive immune system, since they migrate from the epidermis towards the lymph node where they prime naïve T cells. A small immunohistochemistry study on VSCC with unknown HPV status (*n*=5) showed that VSCC were less infiltrated by $CD1a^+$ LCs than healthy vulvar tissue.[48] What this means in the context of VSCC is unclear, but already in vHSIL the epithelial cell nests comprise lower numbers of $CD1a^+$ and $CD207^+$ DC than healthy tissue

and this corresponds with a lower $CD8^+$ T cell infiltrate.[49, 50] The intraepithelial infiltration with $CD1a^+$ DCs is inversely correlated with the grade of VIN.[51] One explanation offered for this was the inhibition CCL20 secretion through E6 and E7 expression.[52] Interestingly, also the dermis of LS is less infiltrated with $CD1a^+$ DC/LCs, and this lack of infiltration is even more pronounced in VSCC that developed in the background of LS.[48, 53] The decrease in intraepithelial $CD1a^+$ DC/LC numbers is paralleled in overall T cell infiltration.[54] Thus, non-malignant vulvar lesions and VSCC display low numbers of intraepithelial immature $CD1a^+$ cells and $CD8^+$ T cells.

In addition to the lower number of APCs detected in VSCC, the function of the remaining APCs may have been altered towards a more immune suppressive phenotype. Cancer cells have been shown to inhibit DC maturation in vitro via the secretion of IL-6 and PgE2.[55, 56] Furthermore, both subtypes of VSCC are known to secrete receptor activator NF-*k*B ligand (RANKL). RANKL can inhibit DC maturation, based on differences in the expression of CD80, CD83, CD86, and HLA-DR as well as their reduced IL-12 production and increased IL-10 production *in vitro,* suggesting that they have become tolerogenic DC. The addition of osteoprotegrin (a RANKL inhibitor) improved DC maturation, suggesting that RANKL could be an interesting therapeutic target to prevent immune escape of VSCC. [57] Furthermore, VSCC expresses indoleamine 2,3-dioxygenase (IDO).[58] This molecule not only suppresses T cell responses [59] but also blocks the maturation of DCs.[60] Notably, VSCC expressed IDO is an independent prognostic factor for worse overall survival in VSCC[58] but it remains to be investigated if this holds true for both subtypes of VSCC.

ADAPTIVE IMMUNE RESPONSES IN VULVAR LESIONS

Two studies showed that $CD4^+$ and $CD8^+$ T cells co-infiltrate the cancer cell nests and stroma of primary VSCC tumors. Furthermore, the magnitude of this T cell infiltration positively correlated with the expression of HLA class 1.[61] In many epithelial cancers, including HPVpos OPSCC and HPVneg OPSCC, a dense infiltration with $CD8^+$ T cells is associated with improved survival.[18, 26, 62, 63] However, a strong infiltration with T cells seemed not related to the prognosis of VSCC[64] which led to the hypothesis that the tumor infiltrating lymphocytes (TILs) had become anergic.[64] Indeed, the functional status of the T cells could make a difference and the expression of the T cell function suppressing enzyme IDO was shown to have a negative impact on survival in one study of VSCC[58], but this was not confirmed in another large study.[61] Also the earlier reported expression of PD-L1 may restrain the function of TIL.[46] Therefore, the functional status of killer lymphocytes was analyzed using single stains for CD56 (expressed by NK/NKT cells) and granzyme B (GrB, for the cytotoxic status). $CD56^+$ lymphocytes were mostly detected in the epithelium whereas GrB^+ cells were predominantly found in the stroma. Importantly, the number of activated $CD56^+GrB^+$ intraepithelial lymphocytes significantly correlated with longer overall survival in VSCC.[65]

Another study in HPVpos VSCC and it's precursors corroborated these findings.[65] Thus, rather than the number of infiltrating lymphocytes, their functional activity is key for clinical impact. Indeed, a detailed study on T cell infiltration, the expression of *Tbet* (for IFN-γ producing T cells) and Foxp3 (for Tregs) and the co-inhibitory molecules programmed cell death 1 (PD-1), T cell immunoglobulin mucin 3 (TIM3), and the natural killer cell lectin-like receptor A (NKG2A) to identify activated T cells, confirmed that vHSIL and HPVpos VSCC are intensely surveyed by activated $CD4^+$ and $CD8^+$ T cells.[40] In vHSIL a dense infiltration with stromal activated $CD8^+TIM3^+$ T cells and stromal activated $CD3^+NKG2A^+$ T cells was related to the absence of recurrences and a prolonged recurrence-free survival.

It is likely that co-infiltration with Tregs blunted the activity of TILs. High numbers of Tregs in vHSIL were negatively associated with time to recurrence, especially when the stromal $CD8^+TIM3^+$ infiltration was limited.[40] In VSCC the number of Tregs is even higher and outnumber the co-infiltrating activated T cells.[40] Potentially, these increased numbers of Tregs in VSCC[40, 58, 61] are the result of tolerogenic DCs in these tumors that support the differentiation of naïve CD4+ T cells into Tregs.[57] Two studies that analyzed the impact of intraepithelial $Foxp3^+$ Tregs in VSCC reported that their number does not influence survival rates.[58, 61] It is not clear if this result was similar for HPVneg VSCC and HPVpos VSCC or what the infiltration with activated T cells was. Furthermore, both studies did not report the impact of stromal Tregs[58, 61], which might be important in view of the presence of activated T cells in the stroma of HPVpos VSCC[40] and the high number of GrB^+ lymphocytes in the stroma of VSCC, with unknown HPV status.[65]

On top of this, the impact of the infiltrating T cells may be lower due to the loss of HLA class I expression in VSCC.[61] HLA-class I was found to be downregulated in 70% of vHSIL patients, 15% of which was caused by loss of heterozygosity (LOH). In the other cases HLA-class I could still be upregulated by IFN-γ.[66] This will prove more difficult for HPVpos VSCC, where HLA-class I downregulation through LOH is found in 25-55.5% of the cases.[66]

Last but not least, the VSCC-infiltrating T cells may be suppressed following interaction with HLA-E, a non-classical HLA molecule expressed in at least 15% of the HPVpos VSCC cases [67], and found to have a negative effect in other epithelial tumors.[68, 69] HLA-E is the ligand for the co-inhibitory molecule NKG2A, which we know is expressed by T cells in VSCC[66] and recently has been shown to function as checkpoint for T cells in several mouse tumor models (van Montfoort et al., *in press*). Furthermore, HLA-E positive tumors show decreased numbers of T cells expressing NKG2A compared to HLA-E negative tumors, indicating a possible negative feedback loop.[40]

In conclusion, the clinical impact of T cell infiltration can't be analysed outside the context of HLA expression, immune suppressive cells and regulatory mechanisms (e.g., expression of PD-1 and NKG2A).

LICHEN SCLEROSUS AND DVIN

Lichen sclerosus is a chronic inflammatory skin disorder that is histologically characterized by a band-like lymphocytic infiltrate below the dermo-epidermal junction. Until now, the immune microenvironment has scarcely been studied in LS and not at all in dVIN. In LS, the immune infiltrate is characterized by high numbers $CD3^+$ T cells, consisting of $CD4^+$, $CD8^+$, and $Foxp3^+$ T cells.[70-75] The numbers of $CD8^+$ T cells and $Foxp3^+$ Tregs were significantly higher in the dermis of LS lesions than in healthy controls[70], A more in depth analysis revealed the presence of high numbers of activated $perforin^+$, GrB^+, and type 1 interferon-associated $CXCR3^+$ cytotoxic cells, suggesting that T cell mediated cytotoxicity occurs in LS.[76, 77] Increased numbers of HLA-DR^+ cells in the dermal infiltrate indicates that these T cells have been activated following cognate antigen recognition.[75] Moreover, gene expression arrays showed that significantly more type 1 immune response associated pro-inflammatory cytokines are expressed in LS when compared to healthy tissue.[70] Together, these data indicate that the microenvironment of LS resembles that of an autoimmune disorder, characterized by a type 1 immune response.

Other indications for a developed T cell response comes from a recent study showing that monoclonal T cell receptor (TCR) rearrangement was identified in 47% of patients with LS.[78] Interestingly, the infiltrates of the lesions with monoclonal TCR rearrangement were dominated by $CD4^+$ T cells, B cells, and fascin (a protein involved in antigen presentation)-positive dendritic cells. Similarly, HPV-negative penile cancers, known to be associated with LS, and differentiated penile intraepithelial neoplasia, showed accumulation of T cells with monoclonal TCR rearrangement.[79] This suggests the presence of a specific antigen in LS and HPVneg VSCC or penile carcinoma to which the immune system can respond.

These findings suggest that LS is an immunologically active lesion, which may give rise to the development of malignancies. Future studies are needed to explore the local immune environment, in particular in relation to clinical outcomes and progression of disease.

MODULATION OF THE LOCAL MICROENVIRONMENT

Until now, the cornerstone of the treatment for VSCC, vHSIL, and dVIN is surgery. This treatment is associated with significant morbidity due to lymph oedema, wound infections, and psychological and sexual problems.[80] However, recurrence rates after these treatments occur in up to 50% of patients.[81] Non-surgical treatments for vHSIL include topical creams and gels, HPV vaccines, and photodynamic therapy (PDT). Understanding of the modulation of the local microenvironment is important as this might unveil clues to novel therapy. An overview of previous clinical trials can be found in table 1.

Imiquimod

Imiquimod activates the innate immune response by interacting with toll-like receptor (TLR)7, expressed by DC, monocytes and macrophages. TLR7 activation induces secretion of proinflammatory cytokines, particularly IFN-γ and also enhances DC maturation and antigen presentation.[82] These cytokines induce polarisation towards a type 1 immune response.[83] Imiquimod is food and drug administration (FDA) approved for the treatment of basal cell carcinoma (BCC) and external genital warts[84, 85], but it can also be effective for the treatment of HPV-induced HSIL of the cervix, vagina and vulva.[86] In a retrospective study imiquimod was found to be effective, with complete responses between 20-81%, and partial responses of 10-75%, as well as and safe for treatment of vHSIL, although long-term response and progression of disease are unknown. The side effects of imiquimod treatment are mainly local adverse effects (itching, burning), which occur in the majority of patients, and flu-like symptoms.[87] Importantly, a randomized controlled trial (RCT) showed objective clinical responses in 81% of vHSIL patients compared to none in the placebo group.[88] In this trial, the clinical response, defined as lesion regression and HPV clearance, was associated with a pre-existing systemic HPV-specific Th1 cell response.[89] Several studies in vHSIL have shown that imiquimod induced T cell infiltration [90-92], including $CD8^+$ T cells.[92, 93] This was associated with expression of CXCR3 and the ubiquitous cytoplasmic protein) MxA, indicative of type 1 IFN-γ signalling.[84] Non-responsiveness to imiquimod was associated with higher levels of intralesional Tregs.[92] Another study showed that clinical responses after imiquimod were associated with increased numbers of intraepithelial $CD1a^+$ and $CD8^+$ cells and a decrease in Tregs in the stroma.[90] In addition, the elevated $CD14^+$ cells found in vHSIL lesions returned to control levels in HPV-cleared patients, in contrast to non-cleared patients. A phase II study of imiquimod followed by therapeutic HPV16 E6E7L2 vaccination indicated that increased $CD4^+$ and $CD8^+$ was associated with clinical response to the vaccine and that relatively elevated Treg levels were observed in the group not responding to the vaccine.[91]

These findings suggest that a combination therapy in which the number of HPV16 specific type 1 T cells are amplified by vaccination and the attraction of immune cells to the lesion site is fostered by local administration of imiquimod will be more successful than monotherapy with either one of the treatments.

Photodynamic therapy

Topical photodynamic therapy (PDT) is an effective and safe nonsurgical treatment option[94] that involves topical application of a non-toxic photosensitizer, which is absorbed into neoplastic tissue. This leads to the production of reactive oxygen species and tumor cell death.[95] The acute inflammation following the clearance of destructed cells is known to contribute to prolonged recurrence free survival in advanced cancers.[95] In addition, the pro-inflammatory effects of PDT might increase DC migration, antigen uptake and maturation. PDT might also increase the immunogenicity of dead tumor cells by exposing or creating

new antigens or by the induction of more effective cytotoxic T cells. Clinical studies in vHSIL have shown varying clinical and histological responses from 20-67%[95, 96], however long-term clinical outcomes are not known.

Immunological studies of PDT in vHSIL have shown a significant $CD8^+$ infiltration in posttreatment vHSIL responders compared to non-responders.[97] In contrast, loss or downregulation of HLA class I was associated with a lack of response to PDT. A phase II trial of sequential imiquimod and PDT in 20 women with vHSIL showed significantly higher numbers of Tregs in the lesions after imiquimod treatment in non-responders, whereas no differences in the infiltration of $CD4^+$, $CD8^+$, $CD68^+$, and $CD1a^+$ cells were observed between responders and non-responders before or after PDT treatment.[92] Interestingly, also in this study a systemic pre-existing HPV-specific immune response was significantly correlated with response to PDT. Together, these studies suggest that the efficacy of PDT is related to immune promoting changes in the microenvironment of treated lesions.

Therapeutic vaccination

The aim of therapeutic vaccination against cancer is to launch an immune mediated attack against cancers by amplifying an anti-tumor T cell response. Therapeutic vaccines stimulate T cells against TSA created by oncogenic viruses or DNA mutations.[98]

VHSIL and HPVpos VSCC express the viral oncoproteins E6 and E7, and expose therefore ideal TSAs for a therapeutic vaccine.[99] In two independent clinical trials it was shown that HPV16 synthetic long peptide (SLP) vaccination of patients with HPV16-induced vHSIL resulted in complete and partial regression of the lesion(s), especially in patients who showed a stronger vaccine-induced HPV16-induced T cell response, irrespective of the immune status of these patients. The first study showed a complete response rate of 47% at 3 months.[100] Post hoc analyses suggested that complete responders had significantly stronger type 1 T cell response. Importantly, a subsequent RCT confirmed that the clinical efficacy of HPV16-SLP vaccination was related to the strength of the vaccine-induced HPV-specific immune response.[89]

Successful treatment of HPV16-induced vHSIL was also achieved with an HPV16 E6E7L2 fusion protein. This phase II study of combination immunotherapy of topical imiquimod followed by HPV16 E6E7L2 vaccine in women with vHSIL showed that there was significantly increased local infiltration of $CD4^+$ and $CD8^+$ T cells in responders, whereas non-responders showed an increased density of Tregs.[91] Similarly, clinical responses after recombinant vaccinia virus encoding HPV16/18 E6E7 vaccination were associated with pre-treatment higher levels of intralesional $CD4^+$, $CD8^+$, and $CD1a^+$ cells compared to non-responders. Although non-responders showed an increase in $CD4^+$ and $CD8^+$, but not $CD1a^+$ cells post-vaccination, their numbers were still lower than the numbers observed in the pre-treatment

samples of responders.[101] These data suggest that therapeutic vaccines can boost the specific T cell response but are only effective in settings where the lesions have the capacity to attract substantial T cell infiltration. The identification of suitable target antigens in HPVneg VSCC is key when vaccination is considered as treatment option.

Topical treatments for dVIN and LS

Interestingly, topical treatment with pimecrolimus induces a clinical response rate in 80% of the LS patients. The response was associated with a significant downregulation of $CD3^+$, $CD8^+$ T cells, $CD57^+$ NK cells, and $CD68^+$ macrophages.[102]

Intriguingly, a recent study in 507 LS patients showed that regular long-term use of topical glucocorticosteroids reduced the risk of developing dVIN or VSCC.[103] Glucocorticosteroids are steroid hormones that inhibit the NF-*k*B pathway, resulting in decreased numbers of transcription factors which regulate the expression of pro-inflammatory genes (e.g. TNF-α and IL-1β) and anti-apoptotic genes.[104] Glucocorticoids have pro-apoptotic properties in lymphoid cells[105], and can selectively inhibit Tregs. Keeping in mind chronic inflammation as a hallmark of cancer[106], inhibition of chronic inflammation by topical steroids may underlie the reduced risk for malignant transformation in LS.

Table 1. Overview of immunotherapeutic clinical studies in vHSIL.

Author	Ref	Treatment	Study design	Phase trial	Patient number	Primary endpoint	Main immunological outcome	Main clinical outcome
Jayne 2002		Imiquimod	RS	2	13	Clinical response	Not investigated.	8/13 CR, 4/7 75% regression
Van Seters 2002		Imiquimod	PS	2	15	Clinical response	Not investigated.	27% CR, 60% PR
Todd 2002		Imiquimod	PS	2	15	Clinical response	Not investigated.	4/15 showed clinical improvement of which 3 had CR
Wendling 2004		Imiquimod	PS	2	12	Clinical response	Not investigated.	7/12 CR or PR
van Poelgeest 2005*	93	Imiquimod	PS	2	17	Immunological assay of responders to imiquimod	Systemic HPV16-specific IFN-γ associated CD4+ T cell immunity associated with CR.	Not investigated.
Le 2007		Imiquimod	PS	2	39	Clinical response	Not investigated.	64% showed CR after 16 weeks, 27% had clinically significant shrinkage.
Mathiesen 2007		Imiquimod vs placebo	RCT	2	32	Clinical response	Not investigated.	81% in the imiquimod group showed CR, 10% PR and no responses in the placebo-group
van Seters 2008	92	Imiquimod vs placebo	RCT	2	52	Clinical response	Higher numbers of intralesional CD1a+ DCs, CD8+ T cells and CD94+ NK cells with imiquimod.	81% of all patients had >25% reduction of the primary lesion

Table 1. Continued

Terlou 2010	45	Imiquimod vs placebo	RCT	2	25	HPV presence (based on p16 IHC)	Lower intralesional CD208+, CD8+, CD4+, CD94+, CD14+ and CD68+ in the HPV cleared patients.	19/25 were HPV-positive before treatment, of which 9 became HPV- after treatment.
Westermann 2013		Imiquimod	RS	2	62	Clinical response	Not investigated.	76% CR, 19% PR, 3% weak PR, 1 non-responder. Recurrent disease in 27%.
Frega 2013		Imiquimod vs cold-knife excision	PS	2	80	Complete response, recurrence rate	Not investigated.	Recurrence rate was lower for imiquimod, relapse rate was higher. The overall CR was inferior in this group.
Kim 2015		Imiquimod	PS	2	9	Clinical response	Not investigated.	4/9 patients had CR, 2/9 PR and 3 were lost to FU.
Winters 2008	95	Imiquimod and PDT	PS	2	20	Clinical response	Responders to imiquimod had higher Tregs before treatment, and more CD8+ T cells after treatment.	Overall response rate 55% by intention treat and 64% per protocol.
Kenter 2009	103	HPV16 SLP vaccin	PS	2	20	Clinical response	Not investigated.	25% CR and histologic response, 35% PR at 3 months.
Daayana 2010	94	x	x	x	x	x	x	x
	Imiquimod and HPV 16 vaccination	PS	2	19	Clinical response	After treatment with imiquimod & vaccination more local infiltration of CD4+/8+ T cells was seen in the responders with lower Tregs.	32% complete response at week 10, 58% at week 20 and 63% at week 52.	x
Van Poelgeest 2016	105	HPV16 SLP vaccine and/or imiquimod	RCT	2	43	Ex vivo detectable HPV16 specific CD8+ T cell response	84% showed systemic vaccine-induced HPV16 IFN-γ associated CD4+ and CD8+ T cell response. No difference was found between the groups.	53% vaccine-induced CR/PR at 3 months. No difference was found between the groups.

RCT = randomized controlled trial, PS = prospective cohort study, RS = retrospective cohort study, CR = complete response, PR = partial response. *van Poelgeest 2005 and Terlou 2010 performed immunological analysis on data from the RCT of van Seters 2008.

CONCLUSION

In this review, we summarized studies that describe the TME in VSCC, and its precursors LS/dVIN and vHSIL (figure 2). The immune infiltrate in VSCC and its precursors is characterized by variable numbers of tumor infiltrating innate and adaptive immune cells. The lesions showed an increase in M2 macrophages and Tregs and a decrease in activated $CD8^+$ T cells compared to healthy controls.[40, 41] In patients with vHSIL, the presence of type 1 immunity is associated with fewer recurrences after treatment and also with better response to topical treatment with imiquimod, PDT, or therapeutic vaccination.[40, 89, 97, 107] Although the number of studies in vHSIL is limited and immune correlates for a treatment response need to be defined, patients with HLA-class I positive lesions, high numbers of infiltrating T cells with

low numbers of Tregs, or pre-existing HPV-specific immunity, may help identify patients more likely to respond to immune therapy. Moreover, in LS high numbers of $CD4^+$, $CD8^+$, and Tregs are found indicating that these lesions are immunologically active and suggest that also the HPVneg VSCC might be potential candidate for future immune therapies.[70-75]

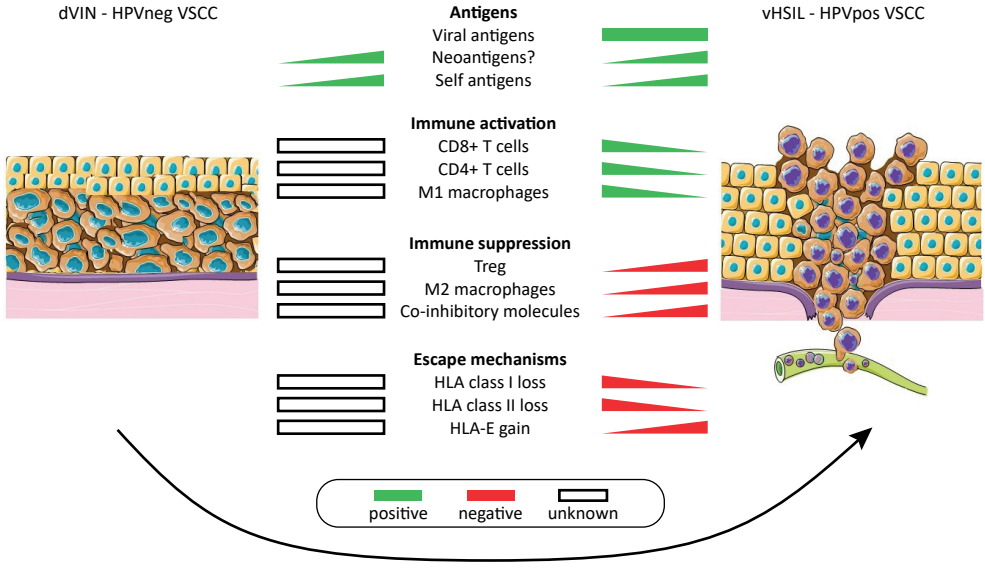

Figure 2. Overview of current literature about tumor microenvironment in VSCC and its precursors. The immune infiltrate in VSCC and its precursors is characterized by variable numbers of tumor infiltrating innate and adaptive immune cells. HPVpos VSCC's precursor lesion is vHSIL, and as progression occurs, these lesions show an increase in M2 macrophages, regulatory T cells, and a decrease in activated CD4+ and CD8+ T cells. Cancer may escape the immune response due to downregulation of HLA class I and II molecules on the cell surface and increased expression of co-inhibitory molecules. Literature on HPVneg VSCC and its precursor dVIN is scarce. Illustrations of lesions created from adapted images of Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License. Treg = regulatory T cell, HLA = human leukocyte antigen.

EXPERT OPINION

Tumors can exploit several immunosuppressive mechanisms to escape from the immune system, by HLA class I loss, or by creating an immune-suppressive TME, by attracting Tregs and suppressive myeloid cells as well as by secretion of immune suppressive cytokines such as TGF-β or IL-10.[98] In addition, ligation of co-inhibitory molecules expressed by T cells, including cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), PD-1, and NKG2a, may occur. Overexpression of the ligand of PD-1 (PD-L1), is associated with poor clinical outcomes in many immunogenic cancers.[108] Monoclonal-antibody-based therapies targeting CTLA-4 and/or PD-1 (checkpoint blockade) have yielded significant clinical benefits including durable responses to patients with different malignancies. Blocking of PD-1/PD-L1 rebalances the host-tumor interaction and is successful in the treatment of many tumor types.[109]

Cancers responsive to checkpoint inhibitors may be those with abundant tumor specific target antigens, generated by a high frequency of mutations that are recognized as non-self-peptides by $CD4^+$ and $CD8^+$ T cells.[110-112] Studies on the expression of immune checkpoint molecules in VSCC are scarce. A recent study in OPSCC showed that the percentage of CTLA-4 and PD-1/PD-L1 expressing T cells was higher in TILs compared to peripheral blood mononuclear cells from patients.[113] Recurrent HPV-associated tumors showed a similar composition of T cell subsets and checkpoint molecule expression as found in HPV-independent tumors. Not to our surprise, blockade of PD-1 /PD-L1 gave similar results in HPVpos- and HPVneg OPSCC.[32] The absence of a better result in recurrent HPV-associated OPSCC is likely because these are the ones lacking an HPV-specific T cell response.[114] Therapeutic HPV16 vaccination induced an immune response which was associated with clinical response in patients with HPV16-induced vHSIL.[100, 107] However, it is unlikely that therapeutic vaccines will be able to overcome the immune-suppressed microenvironment in established and advanced cancers without co-treatment. Indeed, a recent phase I/II study in HPV16-positive cancers showed that HPV16-SLP vaccination in combination with the PD1 antibody nivolumab resulted in a response rate of 36% in OPSCC patients, compared to 16% for nivolumab monotherapy in $p16^+$ OSCC patients.[32, 33] Few studies showed the expression of PD-L1 in a subset of HPVneg VSCC.[46, 115] The presence of $CD8^+$ T cells has been shown to be pivotal for the prediction of response to PD-L1 checkpoint inhibitors in melanoma.[116] Although VSCC is highly infiltrated by $CD8^+$ T cells, high numbers of just $CD8^+$ T cells were not associated with survival.[61] However, three different studies showed that these cells needed to be activated before an positive impact on clinical outcome was detected in early stage VSCC.[65] PD-L1 expression is also found on cervical cancer cells, but not of prognostic value.[117] Besides by tumor cells, PD-L1 is also expressed by M2 macrophages[56], which are frequently found in HPVpos VSCC[41], melanoma and several HPV-associated tumors.[118-121] Importantly, PD-L1 positivity of peritumoral immune cells is an independent favorable prognostic factor for survival[122], probably because it's expression is a telltale for the local production of IFN-ɣ by activated T cells. Further effort must be made to determine the value of this and other checkpoints as predictive biomarkers in both types of VSCC.

As previously mentioned, Tregs are an immunosuppressive cell type in the TME, and some of them are shown to react to tumor antigens.[123] Tregs may be inactivated by treatment of patients with cyclophosphamide, or by inhibition of IDO[124], but also by antibodies against CTLA-4.[125]

Recent clinical data have clearly demonstrated that human cancer cells express antigens that are newly created by mutations in the DNA encoding normal proteins (neoantigens). These mutated cancer proteins can be processed into peptides and presented on the surface of tumor cells leading to immune recognition *in vivo* as "non-self" or foreign by autologous tumor-specific T cells.

A few studies reported a higher incidence of somatic mutations in HPVneg VSCC compared to HPVpos VSCC, correlating with increasing grades of neoplasia.[126] *TP53*, *CDKN2A* and *HRAS* were most frequently mutated, but the full range of mutations still needs to be established as current studies focused on these known hot-spots.[11, 127] In some cancers, the expression of foreign/mutated antigens correlates with increased numbers of TILs and better with clinical outcome.[128, 129] Moreover, high mutational load in tumors is associated with sensitivity to immune checkpoint inhibition and better clinical outcome.[130] These developments are highly promising and open the opportunities for precision medicine in patients with VSCC, including vaccination. On the basis of landmark preclinical studies that identified immunogenic mutations and showed survival benefit upon neoantigen vaccination in prophylactic and therapeutic settings, clinical phase I/II neoantigen cancer vaccine trials are currently ongoing in metastatic melanoma (NCT 03300843, NCT 01970358, NCT 02897765), glioblastoma (NCT 02287428), and pancreatic cancer (NCT 03122106).

Combination treatment of vaccination with checkpoint inhibitors, chemotherapy, or modulation of the microenvironment by PDT or radiotherapy has already shown clinical success in different cancer types.[131] Adoptive cell therapy (ACT) makes use of infusion of tumor-specific T cells into patients with advanced cancers. Currently, phase 1 clinical trials have been initiated with neoantigen-based ACT in patients with myelodysplastic syndrome (NCT03258359) or in patients with glioblastoma, non-small cell lung cancer, ovarian, and breast cancer (NCT03412877). In the light of these developments, it is to be expected that HPV16 oncoprotein-based and neoantigen-based vaccination, in combination with chemo- or radiotherapy, checkpoint blockade and/or ACT will become available for the treatment of advanced VSCC.[132, 133]

By unraveling the VSCC mutational and immune landscape, current treatments can be improved, and new immunotherapeutic interventions can be designed for HPVneg VSCC and HPVpos VSCC and its precursors. Future studies should aim at whole genome sequencing in combination with in depth analysis of the immune microenvironment, not only with respect to type and numeric distribution of innate and adaptive immune cell types, but also to their functional characteristics.

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