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Immunotherapy of Human Papilloma Virus Induced Disease

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Abstract: Immunotherapy is the generic name for treatment modalities aiming to reinforce the immune system against diseases in which the immune system plays a role. The design of an optimal immunotherapeutic treatment against chronic viruses and associated diseases requires a detailed understanding of the interactions between the target virus and its host, in order to define the specific strategies that may have the best chance to deliver success at each stage of disease. Recently, a first series of successes was reported for the immunotherapy of Human Papilloma Virus (HPV)-induced premalignant diseases but there is definitely room for improvement. Here I discuss a number of topics that in my opinion require more study as the answers to these questions allows us to better understand the underlying mechanisms of disease and as such to tailor treatment.

Keywords: Innate immune signaling, CD4 T cells, interferon-gamma, macrophages, cervical cancer, oropharyngeal cancer, adoptive cell transfer.

WHAT DO WE KNOW ABOUT THE IMMUNE RESPONSE TO HPV?

In a recent review of the literature I argued that HPV can be classified into a group of persistent DNA viruses with low antigenic drift, which includes Epstein Barr virus (EBV), Cytomegalovirus (CMV) and Hepatitis B virus (HBV) for which the presence of a virus-specific T-cell response is important for the final outcome of infection. The control of these viral infections require an effective T-cell response comprising both virus-specific $CD8^+$ CTL and $CD4^+$ IL-2/IFNγ-producing Th1 cells. The Th1 cells are required to license the priming of virus-specific $CD8^+$ T cells, to sustain the fitness of virus-specific $CD8^+$ T cells and to modulate the local virus-infected or transformed microenvironment for it to attract $CD8^+$ T cells [1]. It has been long known that T cells, and $CD4^+$ T cells in particular, play an important role in the protection against HPV [2]. In an attempt to put the knowledge about HPV-specific T-cell response in health and disease as well as how it may affect therapeutic vaccination into perspective, the most recent literature was reviewed [3]. Summarized, in healthy individuals one can detect circulating HPV16-specific $CD4^+$ type 1 (Th1) and type 2 T helper (Th2) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) that are reactive to a broad array of epitopes in the viral early (E2, E6, E7) and late antigens (L1) and are able to migrate to areas where viral antigen is presented [4-8]. Furthermore, spontaneous regression of an HPV-induced lesion is associated with the presence of circulating CD4+ and CD8+ T cells specific for HPV early antigens and coincident with the infiltration of the lesion by $CD8^+$ CTLs and $CD4^+$ T cells that outnumber $CD25^+$ regulatory T cells (Tregs) [9-14]. Moreover, in patients with progressive disease the presence of an HPV-specific Th1 response is associated with a better clinical course as well as with a better response to treatment [15-18].

However, in most individuals the presence of HPV-induced progressive disease corresponds with a non-demonstrable or weak T-cell response to the HPV early antigens in the blood [4,5,19-21]. This lack of reactivity is also reflected locally as HPV-specific T cells can be detected in some high-grade squamous intraepithelial lesions (HSILs) and among about one third of the populations of tumor-infiltrating lymphocytes (TILs) in patients with cancer [19,21,22]. The functional activity of these local HPV-specific T cells are likely to be suppressed as TILs may lack cytotoxicity [23] and/or express co-inhibitory molecules such as Programmed Death-1 (PD-1) [24] as well as CD94 and NKG2a [25] at their cell surface. Local immune suppression is also evident from the loss of locally present IFNγ [26-28] and an increase in IL-10 [26,28,29] that is also detected in the serum [30]. In addition, T cells expressing TGFβ have been detected in HPV-induced lesions [26]. These cytokines may directly suppress HPV-specific immunity since IL-10 can potently inhibit the production of pro-inflammatory cytokines and TGF-ß has a potent negative effect on the proliferation and Th1-differentiation of T cells [31]. Notably, IL-10 producing HPV-specific Tregs, highly capable of inhibiting the proliferation and cytokine (IFNγ and IL-2) production of recently activated naïve $CD4^+$ T cells, Th1 cells and CTL, have been isolated from premalignant tissues and cancer [19,32] indicating that part of the local immune suppression may come from a erroneously polarized HPV-specific T-cell response. The number of Tregs are increased in HPV-induced tumors [26,33] probably attracted by tumor-produced CXCL12 [34]. Inside the tumor, these cells can have a major clinical impact since the ratio between tumor-infiltrating $CD8^+$ T cells and $Foxp3^+$ Tregs proved to be an independent prognostic factor in cervical carcinoma [35]. Increased number of Tregs are also found in tumor draining lymph nodes [36].

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In view of this, treatments that attempt to increase the level of strongly activated local HPV-specific type 1 Th-cells and CTL through local immune stimulation, vaccination or the adoptive transfer of HPV-specific T cells are logical.

A GLEAM OF SUCCESS; WHERE FAILURE IS DEFAULT

Stimulation of local immunity by the use of imiquimod (Aldara®) applied to the surface of the epithelium results in type I interferon signaling of keratinocytes and a strong infiltration of the treated tissue by CXCR3 (Th1) lymphocytes [37]. Treatment of HPV-induced high grade lesions of the vulva (VIN3) resulted in viral clearance, normalization of immune cell infiltrate to the level in healthy tissue and a complete regression of the lesion in a substantial number of patients [38,39]. The imiquimod-induced complete regressions were associated with the presence of circulating HPV-specific Th1 cells [18], suggesting that HPV-specific immunity plays a role in the success of this treatment. Non-responsiveness, however, was associated with the local presence of Tregs [40]. An HPV16 synthetic long E6 and E7 peptide vaccine (HPV16 SLP) and a L2E6E7 fusion protein vaccine (TA-CIN), both aiming to induce HPV-specific $CD4^+$ and $CD8^+$ T-cell responses, had clinical success in VIN3 patients either by itself or when given in combination with imiquimod pretreatment [41-43]. The aggregated immunomonitoring data of these vaccine trials paint the picture that clinical success was achieved when strong HPV-specific IFNγ-associated T-cell responses were induced and when locally the number of infiltrating $CD8^+$ and $CD4^+$ T cells was enhanced. At the other hand, non-responsiveness was associated with weaker and HPV16-specific T-cell responses to lower number of epitopes as well as vaccine-mediated increases in the numbers of circulating HPV-specific Tregs and an increased density of Tregs in the lesion [41-43]. The HPV16 SLP vaccine was also capable of inducing significant T-cell responses in patients with cancer [44,45], albeit not to the level observed in patients with high grade vulvar disease and without clinical reactivity. Notably, in a small study two cases with early recurrence showed an increase in HPV-specific Tregs after vaccination [45]. Neither with these vaccines nor with any other vaccine has therapeutic vaccination resulted in the cure of patients with well-established large premalignant HPV-induced lesions or tumors [3]. In analogy to the successes obtained with treating melanoma patients [46,47] adoptive transfer HPV-specific T cells into patients with HPV-induced cancer is now under consideration. The first results in my laboratory suggest that it is possible to consistently obtain HPV-specific effector T cells from patients with cervical cancer under full GMP conditions (van Poelgeest & van der Burg, unpublished observations). Accumulated data of studies on the local environment reveals the presence of different types of immune evasion mechanisms which may hamper the efficacy of current therapies. This advocates combining current strategies with other modalities. For instance, one may combine current vaccines with stronger adjuvants such as IFNα to selectively boost the Th1 response. In addition, combinations with chemotherapy not only to lower tumor load but also to get rid of Tregs and/or other immune suppressive cells can be applied. Other strategies may include the infusion of blocking antibodies against co-inhibitory molecules or the adoptive transfer of HPV-specific T cells and combinations between all of these [3].

QUESTIONS WAITING TO BE ADDRESSED

The insights into the interactions between the immune system and HPV as well as the diseases it causes has allowed us to begin to understand why current immunotherapeutic efforts are successful or fail. Yet there are still many nagging questions that come to mind. In my opinion these are worthwhile to answer as it may help us to better understand this disease and as such the treatment required at each stage of disease.

The first question revolves around the relative importance of either CD4+ T cells or $CD8^+$ T cells in the protection of the host. The well-known role of $CD4^+$ T cells in virus infections is to promote B-cell antibody production and to promote the priming, licensing and sustainment of function of virus-specific CTLs. Virus-specific $CD4^+$ and $CD8^+$ T cells are readily detected with the new state-of-the-art immunomonitoring technologies. The measurement of HPV16-specific T-cell immunity has generally been more difficult than that for other viruses. The detection of HPV16-specific CD8+ T cells have been found to be notoriously difficult and required at least one round of *in vitro* stimulation before detection. Positive responses were found in HPV16-positive patients at all stages of disease but not in healthy subjects [48-51]. In addition, such responses are more often found in HVP16-positive women without CIN than with CIN [5,8]. HPV16-specific $CD4^+$ T-cell responses can be detected directly ex-vivo in the blood of both healthy individuals and in patients at different stages of disease [4,52,53]. In healthy individuals the magnitude of the HPV16-specific $CD4^+$ Th1 response is in the range of that detected against influenza virus [53]. This poses the question whether the response of $CD4^+$ T cells to HPV is more important than $CD8^+$ T cells in the protection against developing HPV-induced lesions. Strong $CD4^+$ T-cell mediated protection against viruses has been described in several animal models of infection [54]. In example, $CD4^+$ T cells controlled acute infection and reduced the number of cells latently infected in the absence of CD8+ T cells or B cells with murine gammaherpesvirus infection [55]. Similarly $CD4^+$ IFNγ-producing T cells protected against a lethal challenge of genital herpes simplex virus type 2 in $CD8^+$ T-cell and B-cell deficient mice [56]. Furthermore, $CD4^+$ Th1 cells were capable of protecting the host *via* the production of IFNγ and direct cytotoxicity in infections with West Nile virus or Friend retrovirus [57,58]. There is no animal model for HPV16 infection but in the successful immunotherapy of HPV16-induced vulvar lesions there is a role for HPV16-specific $CD4^+$ IFNγ T-cell responses while CD8 T-cell reactivity again was much lower and did not show correlation with disease control [18,41-43]. In addition, only certain HLA class II alleles are consistently positively or negatively associated with disease progression in epidemiological studies [101,102]. This sustains the notion that Th1 cells may form the major protective force at this stage. If so, one may ask how these HPV16-specific Th1 cells accomplish this control. Epithelial cells can upregulate HLA class II after exposure to IFNγ whereas the majority (>80%) of cervical carcinomas constitutively express HLA class II [59]. As a consequence $CD4^+$ T-cells can recognize

their cognate HLA class II presented epitope. $CD4^+$ T cells are known to directly recognize virus infected epithelial cells to mediate viral control, partially *via* the killing of these cells [60,61]. Do HPV16-specific $CD4^+$ T cells directly interact with infected keratinocytes? In principle they can but this topic still awaits further study. In addition, $CD4^+$ T cells may indirectly affect infection through the IFNγ-mediated activation of innate immune cell populations. On the other hand, the protection against a progressive clinical course of HPV-induced tumors is more likely to depend on $CD8^+$ T-cell immunity [33]. These tumors are known to down regulate HLA class I. Furthermore, the ratio between $CD8^+$ and regulatory T cells is an independent prognostic factor [35]. There is no direct relation with survival and the number of $CD4^+$ T cells although IFNγ-producing HPV-specific $CD4^+$ can be isolated from a high number of tumors [21,22]. Potentially, they may also help in attracting the $CD8^+$ T cells [62].

The second question concerns a topic that I call the late-early antigen disparity. A number of studies have assessed HPV16 L1-specific $CD4^+$ T-cell reactivity in healthy controls and in patients at different stages of disease with the outcome that the majority of controls and patients displayed proliferative T-cell responses [63,64] associated with the production of IFNγ [65,66]. In a direct comparison, the T-cell response to the early antigens was less frequent and generally not associated with the production of IFNγ in patients with HSIL or cancer [66]. At the one hand this suggests that the HPV16 L1-specific $CD4^+$ T-cell response is not associated with protection against HPV16 infection and associated disease. A notion that is confirmed by the observation that preventive vaccines, albeit capable of inducing strong L1-specific T-cell responses [66,67], are not capable of resolving existing infections [68]. In addition, most of the patients with cancer display L1-specific Th1 cells [66] and L1 is clearly present in cervical cancer cells [69]. On the other hand, it indicates that patients do not have intrinsic problems to mount a strong Th1 type of response to HPV16 suggesting that the presentation of late and early antigens to the immune systems differs with respect to the stimulatory context. As professional antigen presenting cells (APC) sit within the epithelium where their dendrites protrude the layers of epithelial cells it is logical to assume that upon uptake of free viral particles enough L1 protein is available for the processing machinery to allow the production of viral epitopes that can be presented in sufficient quantities to T cells [70]. The early antigens can only be produced after successful infection of the cells in the basal cell layer. These antigens then have to be released to the immune system. Evidently, this does happen considering the high percentage of healthy individuals mounting a response to these antigens [4,52,53]. However, there is a substantial amount of data suggesting that many of the patients with HPV16-induced disease do not develop a $CD4^+$ T-cell response to the early antigens, unless invasive events occur [16,19,20,71]. The question that arises is why some individuals fail to produce an effective T-cell response to the early antigens of HPV but are capable to respond to the late antigen? Potential non mutually exclusive explanations for this phenomenon that come to mind are: 1) This is due to a lack of sufficient amounts of early antigen processed and presented for instance because only a few cells are productively infected, 2) There could be a difference in the immunological context in which these antigens are presented by APCs to T cells, 3) There could be differences in the genetic make up of individuals resulting in less optimal response of the infected keratinocyte to battle HPV. Unfortunately, there is no data available on the immunological response of primary keratinocytes after a genuine HPV infection. A study in which uninfected primary keratinocytes were compared to HPV16 or HPV18 persistently infected primary keratinocytes revealed that persistently present HPV does not strongly affect the expression of the different virus-sensing pathogen recognition receptors expressed by keratincoytes. However, persistent infection was associated with downregulation of: a) components of the antigen presenting pathway, b) the production of antivirals such as type I interferons, c) the production of pro-inflammatory and chemotactic cytokines, and d) components downstream of the pathogen recognition receptors. Notably, many of the downregulated genes are found in a network strongly interconnected by IL-1β, a cytokine crucial for the activation of adaptive immunity [72]. Persistent infection also results in downregulation of genes that normally respond to interferon stimulation [73]. One can imagine that small polymorphisms within all these components may determine the innate inflammatory response of the keratinocyte. Hence, it may less effectively suppress viral replication and/or result in a lower production of cytokines and chemokines to attract the adaptive immune system. Not only will it be important to unravel the molecular pathways involved in the keratinocyte's response to HPV infection but also potential alterations in these pathways that may determine the efficacy of these pathways. It has been suggested that HPV also suppresses T-cell activation *via* the regulation of the function and migration of APCs present in the epithelia as upon pseudo infection of Langerhans cells with HPV16L1L2 virus-like particles the phenotypical and functional maturation of Langerhans cells was suppressed [74,75]. The overall contribution of this effect should be investigated as many healthy and diseased individuals do mount L1-specific immunity suggesting only a minor effect on T-cell priming. In my opinion, there a no apparent defects in the immune system of patients who develop HPV-induced malignancies. Rather these patients are more sensitive to the immune evasion strategies of HPV resulting in a failure to properly stimulate a protective T-cell response to the early antigens of HPV.

The third question relates to the location of HPV-induced malignancies and HPV-specific immunity. An interesting category of tumors in this respect are the squamous cell carcinomas that arise in the head and neck region (HNSCC). Similar to many other tumors, the presence of a strong T-cell associated immune response is also a strong prognostic feature for good clinical outcome in HNSCC. Especially, patients of whom the tumors are infiltrated by high numbers of $CD8^+$ T cells over Tregs display better survival [76-79]. Notably, all most all HPV16+ HNSCC are located in the oropharynx (OSCC) and the majority of these arise in the tonsils. In general, these tumors are heavily infiltrated by immune cells [76-78]. The prognosis of HPV16-induced OSCC is much better than that of their HPV16-negative counterparts as the HPV16+ tumors respond better to chemotherapy and combined chemo-radiation therapy. The

few reports on HPV16-specific T-cell immunity in HNCSCC describe elevated levels of circulating HPV16 E7-specific $CD8^+$ T cells [80] and the presence of IgG antibodies to the E6 and E7 oncoproteins in HNSCC patients with high viral load [81]. Although these antibodies are not expected to exert any anti-tumor effect, their presence indicated the active priming of an underlying helper T-cell response. We recently performed a study of the local and systemic immunity in patients with HNSCC and showed that the majority but not all HPV16+ OSCC were infiltrated by functionally different T-cell subsets ($CD8^+$ T cells, helper T cells, regulatory T cells) that were reactive to HPV16 [82]. In some cases the HPV16-specific effector T cells were highly active *in vivo*, as reflected by their capacity to respond to immunological stimulation directly ex-vivo after isolation from the tumor (Van der Burg, unpublished data). Although the group of patients studied so far was still small our data suggested that HPV16-specific T cells were more often locally present in patients with HPV16-positive OSCC than in patients with HPV16+ cervical cancers, in which about one-third of the tumors contained HPV-specific lymphocytes [22]. We speculated that the location of the disease may play a role in this as the majority of OSCC are located within the lymphoid tissue of the tonsils providing ample opportunities for the immune system to respond. This would fit with the outcome of our studies on HPV-specific T cells in metastasis-positive cervical tumor-draining lymph nodes suggesting that the great majority of tumor-draining lymph nodes contain HPV-specific T-cell reactivity ([21,22] and van Poelgeest & van der Burg, unpublished data).

Despite this it is only 30-40% of the patients with either HSIL or cancer who develop an HPV-specific T-cell response that can be measured in the blood. In HSIL the presence of circulating HPV-specific T cells might be explained by the repetitive destructive unsuccessful treatments and persistence of the lesion of those patients displaying T-cell reactivity [19]. One can envisage that the constant present of antigen in combination with inflammation-inducing invasive treatments may result in the activation of an adaptive immune response. In cervical cancer patients the detection of an immune response was related to the depth of invasion of cervical cancer [16]. Is it just the sheer fact that a tumor penetrates normal tissue that allows an immune response to develop? Or is deep invasion associated with a process that creates inflammation allowing the immune system to respond? These questions are important to address as this may lead to new targets for therapy. A clue for specific inflammation at the site of invasion may come from one of the best studied chemokines able to attract myeloid cells and activated $CD4^+$ and $CD8^+$ T cells, which is CCL2 also known as monocyte chemotactic protein-1 (MCP-1) [83]. CCL2 is secreted by many immune cells and can also be produced by tumor cells. A good number of cervical cancers were shown to produce CCL2 and – of interest to the above question – it is produced especially at the epithelial-mesenchymal junctions [84-86] suggesting that this may form a key factor in the activation of HPV-specific immunity. Although CCL2 is most known for its attraction of macrophages into tumors, a recent study showed that MCP-1 is required for protective anti-tumor immunity by promoting lymphocyte infiltration into the tumor and subsequent cytokine production [87]. The fact that myeloid cells, compared with T lymphocytes, are more often present in tumors may be the result of reactive nitrogen species produced within the tumor. Reactive nitrogen species induces the nitration of CCL2 and this selectively hinders the attraction of T cells [88]. Elevated levels of nitric oxide have been detected in the plasma of cervical cancer patiensts [89]. A profound understanding of the mechanism through which tumor invasion is associated with the attraction of immune cells will foster the development of new strategies.

The last question is related to tumor-associated macrophages. Are these versatile immune cells our friends or foes? Macrophages are present in virtual all tissues. Circulating monocytes form the main source of macrophages during inflammation and trauma, during which the migration of monoyctes from the bloodstream is dramatically enhanced. Macrophages can rapidly respond to danger signals delivered through pathogen recognition receptors and to stimuli generated by innate and adaptive immune cells. Depending on all different cues in the environment macrophages may adapt to phenotypically different populations of cells with distinct functions. Roughly two distinct polarization states are recognized; the classically activated type 1 macrophages (M1) that are associated with tumor rejection and the alternative activated tumor-promoting type 2 macrophages (M2). Notably, macrophages can easily adapt to another state of activation states ranging between the M1 or M2 phenotype depending on the mix of signals in their direct microenvironment [90].

Based on the use of generally one single marker and the fact that macrophage numbers increased with disease progression, most studies suggest that a high number of tumor associated macrophages is beneficial for tumor growth [91]. However, in non-small cell lung cancer the M1 and M2 polarization of the macrophages was determined revealing that a high M1/M2 ratio was associated with improved survival [92]. Also in colorectal cancer the type of macrophage determined survival and activation of T cells [93,94]. In cervical cancer immature and mature dendritic cells as well as macrophages at different levels of differentiation are present in stroma and epithelial compartments of HPV-induced cervical cancer [33,95,96]. The numbers of macrophages are increased compared to premalignant lesions of the cervix [97], which would be expected in view of the production of CCL2 especially by cervical cancer cells and less in the premalignant lesions [86]. Some cervical cancers, but not all, can secrete immunomodulatory compounds such as PgE2 and IL-6 [98,99] that have been shown to steer macrophage differentiation towards an M2-like macrophage [96]. The indications that not the macrophage *per se* but the type of macrophage present may determine tumor infiltration and clinical outcome warrants similar studies in HPV-induced cancers as new therapies are targeting molecular pathways regulating macrophage polarization [100].

In my opinion we are starting to understand the relationship between the immune system, HPV infection and subsequent (pre)malignancies. However, major questions still need to be addressed. The answers undoubtedly will foster the development of new therapies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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