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**Author:** Heusinkveld, Moniek  
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CHAPTER 8

General discussion
GENERAL DISCUSSION
The role of APC in the activation of T cells

In chapter 2 we analyzed the presence of HPV-specific proliferative T-cell responses in the blood of patients with HPV-induced cervical cancer. HPV-specific T cells were found only in a minority (31%) of the patients. T-cell responses were more often detected in patients presenting with a tumor that deeply invaded the surrounding tissue, which is on its own a bad prognostic sign. At that time we hypothesized that by the infiltration of healthy tissue, tumor antigens were delivered to APC not tolerized by the tumor milieu and, therefore, able to appropriately instruct T cells. However in view of the results presented in chapter 5, in which we showed that CxCa can induce macrophage differentiation, I would like to propose an alternative explanation. Macrophages contribute to tumor invasion by the production of metallo proteinases (MMP) which brake down collagen and facilitate infiltration by tumor cells in the surrounding tissue. Therefore, the deeply invading tumors described in chapter 2 could be the tumors that harbour high numbers of MMP-producing M1 or M2 macrophages. In chapter 2 we describe that half of the patients with deeply invading tumors have proliferating HPV specific T cells in the blood. After uptake of tumor cells or cell debris macrophages can present tumor-specific antigens to T cells and elicit T-cell responses. Analysis of the H&E staining of these deeply invading tumors showed that the patient group with T-cell proliferation in the blood had high immune cell-infiltrate in the tumor (Table 1). This suggest the presence of pro-inflammatory M1 macrophages, contributing to local invasion of the tumor but also inducing inflammation and T-cell responses.

In contrast, patients without HPV-specific proliferation in the blood predominantly lacked immune-cell infiltrate in the tumor. One can envisage that the patients without T-cell responses in the blood and low infiltrate density in the tumor (table 1) harbour M2 type macrophages in the tumor. These macrophages are not attracting or inducing T cells but promote tumor invasion and this correlates with the poor prognosis found for this group (chapter 2).

We showed that when HPV-specific T cells were present in the tumor, the T-cell reaction of a wide diversity and involved several CD4+ as well as CD8+ T-cell clones (chapter 3). Ex-vivo, these T cells recognized HPV but not all responding cells produced type 1 cytokines. Addition of TLR-agonist PAM3CSK4 and Poly I:C during antigen-specific stimulation of the T cells increased their production of IFNγ in primary cultures. To stimulate the T cells with antigen we made use of B-LCL as antigen presenting cell. Although B cells can present antigen, they are not as the specialized as DC and, therefore, these assays may not have been optimal for testing T-cell function. How important the type of APC is for the strength of activation in antigen-experienced cells is not clear, although it is known that the type of co stimulatory molecules present influence cytokine production [1]. Furthermore, we showed in chapter 5 that naïve responder T cells failed to produce high levels of IFNγ when stimulated with less well differentiated DC or macrophages – obtained when monocytes were cultured in the presence of tumor supernatant - as compared to T cell stimulated with fully differentiated DC. In addition, we observed that established HPV-specific T-cell clones produced less IL-2 upon activation with the above mentioned non-optimal APC (unpublished observations).Apparently the type of antigen presenting cell is important for the activation of naïve but also antigen experienced T cells. Finally, experiments on T-cell activation by peptide loaded monocytes revealed that increased antigen doses resulted in better cytokine production by T cells (Singh
et al., manuscript submitted). Hence, conclusions concerning the functionality of T cells (e.g. like we have called the T cells that did not produce cytokines ‘poised’ in chapter 3) should be drawn with great care.

**TLR-agonists to boost inflammation?**

Addition of TLR-agonists boosted the IFNγ production of the aforementioned cultures described in chapter 3 but not to the same extent in all patients. PAM3CSK4 and Poly I:C were the most promising adjuvant. In these cultures the exact cell type (APC, T cells and in lymph node tissue also B cells) that responded to the TLR-agonist was not dissected.

Several studies report that stimulation of TLR modulates T-cell responses after TCR triggering [2]. At the T-cell population level, TLR-stimulation enhanced IFNγ production by T cells but also abolished regulatory T-cell function [2]. So far, these studies were performed on unselected CD4+ T cell populations. In our study on functional TLR expression by T cells we made use of isolated HPV-specific T cell clones and conducted many in vitro experiments. Analysis of TLR expression by patient derived tumor-antigen specific CD4+ T-cell clones -including helper and regulatory T-cell clones- revealed that human T cells express a number of different TLRs at the mRNA level and that the TLR expression pattern differed per CD4+ T-cell clone, independent of their function. When these CD4+ T-cell clones were tested in various activation (proliferation, cytokine production) and suppression assays no consistent gain (proliferation/IFNγ production) or loss in function was observed due to the presence of TLR-agonists. This suggests that TLR may not play a major role in direct stimulation of antigen experienced and fully polarized CD4+ T cells (unpublished observations).

The effect of several TLR-agonists on the activation of purified APC was investigated in vitro in chapter 5. Interestingly, the two agonists PAM3CSK4 and Poly I:C - showing the most promising activation of T-cell cultures in chapter 3 - were the least promising agonists in these assays (chapter 5a, figure 2 and not shown). Addition of TLR-agonists to mo-DC resulted in upregulation of co-stimulatory molecules at the cell surface, but Poly I:C as well as PAM3CSK4 failed to induce the production of IL-12. However, when Poly I:C was added to a less pure culture of mo-DC (derived by plastic adherence) splendid production of IL-12 was detected. These cultures are contaminated with T cells which may have provided additional signals via CD40-L or IFNγ [3,4] suggesting that Poly I:C in the presence of a second signal is a strong agonist (unpublished data).

This notion is sustained by our studies using skin-explant cultures as described in chapter 6. Poly I:C proves to be the strongest activator of dermal APC. Here too, other cell types are present that can provide synergistic signals to the local APC. Interestingly, R848 -the agonist of TLR7 and closely related to Imiquimod- did not induce any measurable activation or migration in the skin-explant model described in chapter 6. Keratinocytes, which functionally display TLR3 but not TLR7 may have supported the activation of APC by the production of IL-1, type I IFN and other factors ([5] and unpublished observations). Based on chapter 3 and 6, Poly I:C may form an attractive compound to apply locally on tumors or neoplastic lesions. Especially for HPV+ oropharyngeal tumors this could be a good alternative or adjuvant treatment. These tumors develop within a lymphoid structure and thus consist of lymphoid tissue and epithelial cells (e.g. keratinocytes). Since many HPV+ oropharyngeal tumors are infiltrated with tumor-antigen specific T cells, stimulation of local inflammation might tip the balance in favor of an anti-tumor response (chapter 4).
**M2 and myeloid derived suppressor cells (MDSC)**

Tumors attract myeloid antigen presenting cells and interfere with their differentiation. Gabrilovich et al showed in mouse models that tumor-induced myeloid cells suppressed surrounding T cells. These MDSC express GR1+ as well as CD11b, produce iNOS and arginase and are of pre-mature undifferentiated state [6]. MDSC are also found in humans, although the phenotype is less clear and there is a great variety between individuals and tumor types. Human MDSC express CD33, CD11b and IL4Rα, CD15, CD14 although the last 3 markers are not always reported [7]. In chapter 5 we showed that soluble factors produced by CxCa cell lines influence myeloid cell differentiation despite the presence of IL-4 and GM-CSF. Some cell lines only hampered DC differentiation but other cell lines clearly induced a macrophage phenotype. The APC differentiated in the presence of supernatant of CASKI cells expressed less CD1a and higher mannose receptor. Other markers like CD14 or IL-4Ra were not clearly expressed and, therefore, it was difficult to nail down the exact cell phenotype that was induced. The differentiation of these cells into DC was completely restored when IL-6 was blocked. Thus in our experiments IL-6 only hampered DC differentiation and induced higher IL-10 production but did not skew cell differentiation towards macrophages or MDSC.

Furthermore, we showed in chapter 5 that CxCa-produced PGE2 skewed mo-DC differentiation towards APC expressing CD14, CD163 and high levels of the mannose receptor. Based upon receptor expression profile and functional characteristics we called these cells type 2 macrophages. Similarly, Obermajer et al. recently published that the addition of PGE2 to mo-DC cultures induced suppressive cells which they called MDSC [8]. This is a clear example of the confusion and debate that is currently ongoing about the phenotype, function and role of myeloid subsets in human cancers. To complicate matters even further, we clearly found that activation with TLR-agonists or CD40-L of M2 macrophages resulted in phenotypic changes leading to loss of the typical M2 marker phenotype but exertion of their M2 function (chapter 5a supplementary. fig. 1). Thus the phenotype of APC is not always representing the function of the cell, even more because myeloid cells show great plasticity depending on the type of stimulus. Finally, tumors harbour distinct microenvironments that instruct locally present myeloid cells and results in heterogenic populations of myeloid cells within one tumor, both in mice and men [9-11]. These data plead for a less stringent separation of myeloid cell subsets. MDSC or macrophages are not different cell subsets but rather resemble a state of differentiation and activation of developing myeloid cells.

**Chemotherapeutics induce immunological effects**

Chapter 5b describes the preliminary data of studies in which tumor cell lines known to skew monocytes to M2 macrophages are treated with cisplatin or carboplatin. Treatment of these tumor lines with physiological but non-lethal doses of cisplatin resulted in an increased production of PGE2 and IL-6 by the tumor cells and augmented the skewing of monocytes to M2 macrophages. This illustrates that the quantity and the sum of factors released by the tumor cells bears impact on the final outcome. Possibly other factors are produced by the CxCa cell lines as well and contribute to the observed effects. The fact that COX-inhibition in tumor cells prevented the skewing to M2 macrophages illustrates that PGE2 is a key factor in this network.

If patients receive chemotherapy treatment it is likely that a peak dose emerges in the blood followed by a rapid decline. Ideally, all tumor cells die upon treatment but based on
the results of chapter 5b, one can envisage that if tumor cells are resistant and survive they might actually contribute to an increase in the number of local M2 macrophages and as such promote tumor growth. Ferrandina et al. performed a clinical study in patients with advanced CxCa and found that over-expression of COX-2 in the tumor correlated with a poor prognosis upon chemo radiation. This was explained by the fact that COX-2 expression in the tumor made the cells more resistant to apoptosis and thereby to chemotherapeutic treatment [12-15]. However the data of chapter 5b advocates an alternative explanation. An important predictor of response to therapy in several human tumor types is the number and type of T cells present in the tumor [16-21]. Maybe in cervical cancer these two known prognostic factors are related because the type and number of T cells infiltrating could reflect the type of macrophages that are present. High numbers of M2 macrophages correlate with more regulatory T cells infiltrate in human ovarian and GIST tumors [22,23]. Therefore the tumor expressing high regulatory T cell numbers is likely to be the COX+ tumor that induced the differentiation of M2 cells via elevated levels of PGE2 and IL-6 in its micro milieu. Besides the tumor promoting activity of the M2 macrophages, increased suppression of locally present T cells – either directly by the production of IL-10 by M2 macrophages or by the attraction of regulatory T cells - will occur and this is enhanced upon treatment with cisplatin (chapter 5b). This notion is sustained by a study in mice bearing lung tumors. Here, treatment with COX-inhibiting drugs resulted in marked T-cell infiltration in the tumor and delayed tumor outgrowth. The tumor infiltrating APC produced less IL-10 and more IL-12 upon treatment. This indicates that local PGE2 levels altered the APC type in the tumor with effects on T-cell infiltration [24]. Interestingly in lung cancer a poor response to cisplatin therapy is associated with activation of STAT3 protein. IL-6 signaling is known to activate STAT3, suggesting that cisplatin-mediated increase of factors like IL-6 – known to affect DC differentiation - might play a role in the poor response to cisplatin of these patients as well [25].

Unfortunately studies on the simultaneous presence of different T cells types in relation to the infiltrating APC on human tumors are scarce. This makes it difficult to find data corroborating above mentioned hypothesis. Studies in which these parameters are combined may reveal a relationship between these players, especially when analyzed before and after treatment. Currently, mouse tumor models are explored to further dissect the exact role of infiltrating APC and T cells upon in relation to treatment. A major drawback of such models is that only a few tumor lines are suitable to study this. As we have shown in chapter 5, pronounced differences between cell lines exist with respect to their capacity to influence immune cells. Mouse studies using tumor cell lines will poorly reflect the great variety in human tumors and thus their interplay with immune cells.

Notably, platinum based chemotherapeutics can have positive immune-related side effects. In a mouse model, Apetoh et al. showed that oxaliplatin induced immunogenic cell death via calreticulin exposure on tumor cells, thereby, stimulating the induction of an anti tumor T-cell response. Oxaliplatin and cisplatin induced release of HMGB-1 in dying tumor cells that activated APC via stimulation of TLR-4 [26,27]. However, most of these experiments were done with only a few tumor-cell lines and intra-tumoral injection of chemotherapeutics or ex-vivo treatment of the tumor which does not resemble the clinical practice. This mechanism might be relevant in human cancer though since patients with breast cancer carrying a loss-of-function TLR-4 mutation relapsed more quickly after radiotherapy combined with (non-platin
based) chemotherapy [26], arguing for immune-dependent effects of chemotherapy. The data in chapter 5 shows that tumor induced macrophages respond to TLR-4 agonists, as reflected by the production of IL-10. Therefore, it is highly likely that chemotherapy-mediated release of HMGB-1 may not only activate DC but activate locally tumor-promoting macrophages as well. Activation of macrophages would add to the immune-suppressive tumor-promoting micro milieu in the tumor. Thus, platinum-based chemotherapy of solid tumors may both have positive and negative effects on the local anti-tumor immune response. The outcome is likely to depend on the constitution of the population of different APCs present within the tumor. Finally, most patients are treated with a combination of radiotherapy and chemotherapy but the combination of these two treatments is yet not addressed in the former and our own studies. Radiotherapy enhances antigen presentation by tumor cells, induction of tumor antigen specific antibodies and inflammation [28-30] and is, therefore, likely to contribute to the overall immune response as well.

**Blocking COX-2 or IL-6 during treatment**

Blocking the production of PGE2 with COX-inhibitors prevented the induction of tumor promoting macrophages *in vitro* (chapter 5) and, therefore, patients with COX overexpressing tumors may benefit from blocking these enzymes during therapy. A number of studies were performed in several solid cancers in which patients were treated with a COX-2 inhibiting drug during standard therapy. In a recent phase II study COX-2 inhibition favored the clinical outcome in patients with ovarian cancer [31]. Also a daily dose of celecoxib (most clinically used COX-2 inhibitor) effectively prevented (pre-cancerous) colorectal polyp formation. Unfortunately, the latter trial was aborted because of serious adverse cardiovascular effects when patients were treated for several years [32]. Also in non-small-cell lung cancer the addition of celecoxib during a shorter time period to chemotherapy was studied. Although no adverse cardiovascular side effects were reported also no survival benefit was found in two independent studies [33,34]. COX-inhibition in patients with neoplastic lesions was already advocated because the increased chemo-sensitivity of the tumor cells [13-15]. We provide with chapter 5b an immunological rationale for the addition of a COX-inhibitor to standard treatment. Also in combination with therapeutic vaccination, inhibition of COX-enzymes may contribute to a less hostile tumor microenvironment that is more vulnerable for the attack by T cells.

A second strategy to change the tumor microenvironment into an attractive place for pro-inflammatory macrophages and cytotoxic T cells is blocking IL-6. We found that cancer cell derived IL-6 skews DC differentiation towards APC that produce more IL-10 (chapter 4) as well as displayed elevated levels of activated STAT-3 (data not shown). Recently, a study was reported in which chemo resistant end-stage ovarian cancer patients were treated with an antibody to IL-6 (siltuximab). Stable disease was observed in some patients [35]. Concordantly, in a xeno-graft ovarian cancer mouse model blocking of IL-6 resulted in less macrophages infiltrating the tumor [35], indicating that tumor-rejection associated immune-effects can be achieved by blocking IL-6 signalling. In rheumatoid arthritis, a monoclonal antibody to IL-6Receptor is used with great clinical benefit by stopping inflammation upon treatment [36]. Treatment with blocking antibodies to IL-6 can break the autocrine supportive loop of IL-6 producing tumor cells as well as IL-6-mediated suppression of DC differentiation or induction of M2 macrophages. Therefore, addition of IL-6 blocking antibodies to standard treatment or therapeutic vaccination should be considered.
Monoclonal antibody can activate any type of local macrophage

In chapter 7 we describe that blocking growth receptors on tumors as anti-tumor therapy may have unwanted immunological side effects. We showed that tumor promoting M2 macrophages are activated by tumor bound cetuximab although this monoclonal antibody that targets EGFR on tumor cells was optimized to bind FcyReceptors on NK cells. For this, we used an in vitro culture system to differentiate monocytes into M2 macrophages by M-CSF. These macrophages responded to tumor bound antibodies with the production of the immune suppressive IL-10 and IL-8. Although M-CSF is widely used to differentiate human M2 macrophages in vitro, these might not resemble the tumor-supernatant induce M2 as described in chapter 5b. To study the differences, we analyzed M2 macrophage cultures derived by culture protocols with distinct cytokines (chapter 5b). Although these macrophages expressed similar phenotype, the response to stimuli was not equal. For example the cytokine production upon CD40-ligation was poor in the M-CSF cultures whereas the IL-6+PGE2 induced cultures (resembling CxCa cell line supernatant) produced high amounts of IL-10 upon this activation. Taken these together, the experiments described in chapter 7 might not resemble the real tumor associated macrophages as induced by CxCa cell lines (chapter 5b). However (unpublished) control experiments showed that TSN-M2 macrophages -as described in chapter 5- are also activated by tumor-bound cetuximab and not by control antibody, indicating that M-CSF derived M2 macrophages were appropriate cells for these proof-of-principle tests. Of note, if M1 macrophages, that also express FcReceptors, are present in the tumor they will get activated as well and engulf or kill the cells upon binding cetuximab opsonized tumor cells. Therefore, the net outcome of a treatment may again be determined by the immune-cell repertoire that is present in each individual tumor. Based on the results with cetuximab, targeting the cell surface expressed IL6receptor by tocilizumab may also result in macrophage activation. Although it has been shown that this MAb did not activate DCs, no specific experiments with the MAb bound to IL-6R-expressing cells have been reported [37].

Re-polarization of macrophages – a way to treat cancer?

The treatment of cancer may take advantage of therapies that interfere with M2 macrophages, if combined with standard or immunotherapeutic regimens. Therapeutic modalities may attack at several levels; the attraction, the differentiation or the activation of macrophages.

One therapeutic option is to interfere at the level of macrophage attraction and differentiation by abrogation of the PGE2 and the IL-6 and STAT-3 activation loop or other tumor produced cytokines [38,39]. This indirectly affects tumor growth and limits the induction of tolerogenic macrophages as described earlier. It might prove difficult to reach high enough antibody titers to block cytokine levels locally in the tumor, as they often are poorly vascularized at area’s where macrophages tend to accumulate [40]. Care should be taken since as membrane bound antibodies may activate macrophages via cross linking of the FcγReceptors and thereby activate inflammatory cascades (chapter 7).

IFNγ in combination with the CD40-CD40L APC-activation signal effectively reprograms tumor-induced M2-like macrophages into activated IL-12 producing M1 cells (chapter 4). For this to occur in the tumor-microenvironment, it is essential that CD4+ Th1 cells are locally present. The capacity of tumor-specific Th1 cells to directly alter the tumor microenvironment has also been recognized in studies on tissue-infiltrating CD8+ T cells in mouse models. Th1 cells were essential for successful recruitment, local expansion and full effector function of large
numbers of CTL by modulation of the local environment [41,42], and this may have included the repolarization of macrophages. In order to obtain sufficient numbers of tumor-specific CD4+ Th1 cells one may make use of adoptive T-cell transfer protocols or apply strong vaccines [43-46]. These therapies should not only induce cytotoxic T cells but also type 1 polarized helper cells to help shifting the local tumor micromilieu from tumor-promoting and immune suppressive into anti-tumor inflammation.

**Figure 1.** Modulation of tumor associated macrophages. Monocytes leaving the bloodstream and entering the tissue differentiate in APC but this is hampered by tumor produced soluble factors. In the proximity of a PGE2 and IL-6 producing tumor their differentiation is skewed towards M2 macrophages. These macrophages produce VEGF and MMP which support tumor growth but also high IL-10 and low IL-12 suppressing T-cell function and precluding type 1 T-cell induction. However if this M2 macrophage interacts with an IFNγ producing CD40-L expressing CD4+ T cell it can revert into a IL-12 producing macrophage capable of inducing type 1 T cells. Cisplatin or carboplatin treatment of a tumor that produce PGE2 and IL-6 (likely to be a COX-2 positive tumor) can induce higher levels of these factors and results in more M2 skewing. M2 induction by the tumor can be avoided by treating the patient with drugs that block COX-2 enzymes thereby preventing PGE2 production or blocking of the tumor produced IL-6 with MAb to IL-6 or IL-6Receptor. Induction of tumor specific IFNγ producing T cells by therapeutic vaccination or adoptive T cell transfer (ACT) can shift the balance in the tumor towards anti-tumor inflammation by T-cell mediated activation of the local APC. Therapeutic options are depicted in the blue boxes.

**Immunogenic HPV16+ oropharyngeal tumors**

Intriguingly, the incidence of HPV related cancers of the head and neck region are rapidly increasing. Another unresolved issue is how this virus can persist and cause cancer in an immunologic organ like the palatine tonsil of the Waldeyers ring. The palatine tonsils consist
of lymphoid follicles that are covered with squamous epithelium that form crypts [47,48]. The reticulated crypt epithelium, also called lymphoepithelium plays a key role in the initiation of immune responses in the palatine tonsils. Luminal antigens are taken up in the crypts and transported to sub epithelial spaces where they come in contact with lymphoid cells. Not only HPV virus can settle in these crypts and infect basal cells of the epithelium but also a high load of fungi, bacteria and other viruses pass by this tissue at daily basis [47].

In chapter 4 we describe that in 6 out of 8 HPV16+ oropharyngeal cancer patients HPV specific T cells were present in tumors or LN. Although the cohort is very small and the results need to be validated in a bigger cohort, these data suggest that local HPV-specific T cells are more often present or less suppressed compared to HPV16+ cervical cancer [49]. In two patients HPV-specific proliferating and IFNγ producing TIL could be readily detected in a direct ex-vivo analysis of a single cell suspension from a fresh biopsy (without the need for a homeostatic proliferation period). This indicates that a strongly activated tumor-antigen specific T-cell population is present within these tumors (data not shown). In the tonsil, local APC can take up tumor antigens but in parallel may get activated through stimulation of their pattern recognition receptors by normal bacterial and viral flora entering the body. This concomitant activation results in stronger signal 2 and 3 delivery of APC to T cells. We hypothesize that infection and inflammation caused by other pathogens may appropriately activate APC in this lymphoid structure to prime HPV16-specific T cells resulting in the strong T cell response found in these (few) patients. Furthermore I speculate that the type of pathogen co-infesting the tonsil at the moment of T-cell priming might be of importance for the T-cell response that is subsequently induced. This because we showed in chapter 5 and 6 that different TLR-agonists show unique activation patterns in APC resulting in different T cell stimulating abilities.

Lesions in the oral cavity known as leucoplakia progresses in 20% to cancer and studies on the presence of HPV in these lesions showed conflicting results. Although HPV was more often detected in lesions compared to healthy control tissue is was not exclusively detected in lesions. Furthermore the prevalence of HPV in lesions varied from 17%- 68% between studies (reviewed by [50,51]). Whether these lesions are caused by the virus or that the virus just better persists in ulcerated tissue and therefore is more often detected, is not known. Also pre-cancerous lesions of the Tonsil are not studied yet and this is the localization where HPV+ HNSCC develops. Thus whether oropharyngeal neoplasias develop in the same way as cervical neoplasias (progression of CINI, II, III to malignant carcinoma) remains an open question. Studies on the presence of pre-malignant lesions in combination with determination of the present flora in the tonsil and a detailed analysis of local T-cell infiltrate and specificity might elicit some answers to the previous mentioned discrepancies between HPV-induced CxCa and HPV-induced oropharyngeal carcinoma.

We described that circulating HPV specific T cells in HPV+ CxCa (chapter 2) as well as HPV16+ oropharyngeal cancer (chapter 4) proliferate but often lack detectable cytokine production when stimulated in vitro. In contrast to the circulating T cells, HPV-specific tumor infiltrating T cells of most HPV+ oropharyngeal tumors produce type 1 cytokines upon peptide recognition (chapter 4). Also here, the design of the study and the APC used for the test might have influenced the results. However in both test monocytes have presented the peptides and in the patients with HPV-negative tumors 4 out of 7 responses were accompanied by IFNγ showing that monocytes can activate the T cells properly in these experiments.
Oropharyngeal tumor-derived TIL were able to respond to HPV oncoproteins with the production of type 1 cytokines when directly tested after isolation from a digested biopsy (n=2, data not shown) or short culture period (chapter 4). On the other hand we also showed that in addition to HPV16-specific IFNγ and TNFα producing Th1 cells also HPV16 specific IL-5 producing CD4+ Th2 cells, Treg and FoxP3 expressing CD8+ T cells could be isolated out of an oropharyngeal tumor. To understand why these patients, despite the presence of functional tumor-antigen specific T cells, can not fully control their growing HPV16+ tumors also the other tumor-infiltrating immune cells must be analyzed. For instance, one explanation could be that—similar to what is found in breast cancer [16]—CD4+ T cells promote pro-tumor inflammation by M2 macrophages in oropharyngeal tumors. Therefore, in depth analysis of the ex-vivo TIL population as described in chapter 3 for CxCa TIL should be performed including the analysis of Th2 and Th17 populations in this patients with oropharyngeal cancer. Furthermore, single cell suspensions of these tumors could be analyzed not only for lymphocytes but also for the presence and phenotype of the myeloid cells. Finally, analysis of T cells isolated from regressing lesions after therapy might shed light on the role of these cells in the better disease free survival that HPV+ HNSCC patients display.

For the treatment of HPV16+ high grade vulvar intra-epithelial neoplasia (VIN), promising results were obtained with a synthetic long peptide (SLP) vaccine that induced strong and broad Th1 type T cell immunity to E6 and E7 [52,53]. Although patients with HPV16+ HNSCC carcinoma respond surprisingly well to radiotherapy this heavy treatment is not without side effects. It is suggested that the immunogenic properties of these virally induced HPV16+ oropharyngeal tumors contribute to the good response to treatment ([54] further supported by the data of chapter 4). Therapeutic vaccination aiming to reinforce the T-cell response to the HPV16 oncoproteins, thus may form a promising treatment modality. Schiering et al. recently published that tumor specific CD4+ T cells that spontaneously had developed in mice, suppressed the induction of new T cells by vaccination [55]. In view of our data showing that HPV specific T cell responses are present in the majority of the circulating T-cell and TIL- populations of patients with HPV16+ oropharyngeal tumors (chapter 4), vaccination may predominantly lead to boosting of already existing HPV16-specific T-cell responses and increase the number of HPV16-specific Th2 cells and regulatory T cells [56]. Therefore a prospective study on the presence of HPV-oncoprotein specific immunity, the correlation with survival and what happens with these cells upon treatment in patients with HNSCC should be the next step.
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