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**Title:** Deregulation of innate and adaptive immune responses in human papillomavirus infection and cancer  
**Issue Date:** 2015-05-07
Chapter 6

Discussions
The data presented in this thesis revolve around the deregulation of immunity by hrHPV in the early phase of the infection cycle and in HPV-induced cervical cancer.

At the early phase of the infection cycle, HPVs need to avoid immune responses of the host in order to establish persistent infection. Our data show that HPVs achieve this by dampening innate immunity of keratinocytes, the major cell type targeted by HPV. As there is reduced production of danger signals including antimicrobial molecules, proinflammatory cytokines and chemokines by keratinocytes, HPV infection may remain undetected by the immune system in line with the long time it takes to control HPV infection. However, further data presented here show that PRR signaling is not completely blocked by hrHPV. Thus, the activation of innate and adaptive immunity at the site of HPV infection is slowed down but not prevented. This fits with the observation that HPV-infected subjects are capable of mounting an HPV-specific immune response at some point in time. In order for cancers to grow out they need to suppress the local effector cells. Previously a role for local regulatory T cells was found. Furthermore, the tumor microenvironment was shown to contain many M2-macrophages, albeit that these did not seem to have any direct impact on disease progression. When we focused on the role of the PD-1 receptor and its ligands PD-L1 and PD-L2, our data showed that the majority (81%) of the tumors from cervical cancer patients do not express PD-L1. Furthermore, PD-L1 expression was not associated with patient survival. About half of T cells infiltrated in the tumor of cervical cancer patients did not express PD1, suggesting that the PD-1/PD-L1 axis might not play a critical role in T cell dysfunction in cervical cancer patients. Finally, we also presented evidence that during early infection HPV sets the stage for increased proliferation and survival of HPV infected cells by expressing elements of the immune system’s chemokine signaling pathway.
Expression of PRRs in relation to KC differentiation

In view of the notion that stratified squamous epithelia consist of undifferentiated (basal layer) and increasingly differentiated (suprabasal and apical layers) KCs, we charted PRR expression in relation to KC differentiation. For 6 TLRs, similar expression levels were detected in KCs of all differentiation stages: TLR1, TLR2, TLR3, TLR5, TLR6 and TLR10. For 3 TLRs, no expression was detected in any of the differentiation stages: TLR4, TLR7 and TLR8. Our findings concerning expression of these TLRs in KCs are largely in line with previous reports by others. The absence of TLR4 expression in differentiated KCs, as opposed to the detection of TLR4 in differentiated HaCat cells is consistent with work by others showing that TLR4 was only found in HaCat cells, but not in primary human KCs. Interestingly, expression of TLR9 showed striking changes upon KC differentiation, in that it was undetectable in undifferentiated and partially differentiated KCs, while being readily detectable in fully differentiated KCs. Analysis of TLR9 protein expression by immunohistochemistry in sections of human foreskin and exocervical epithelium revealed that TLR9 was not detected in the basal layer of the epidermis but prominently expressed in the upper-spinous and granular layers. Staining intensity for TLR9 increased towards the most apical KC layers. Taken together, our data conclusively show that TLR9 is absent from undifferentiated KCs and that its expression is progressively induced by KC differentiation. These results suggest that the discrepancies in previous studies with respect to the expression of TLR9 in KCs are related to differences in culture conditions, which are known to readily affect the differentiation stage of KCs. There is indeed inconsistency in TLR9 expression data as shown in Hasan et al., because one of the normal KC lines (NHK1) is TLR9 positive, while the second line (NHK2) is TLR9 negative.

High risk HPVs do not directly impact on PRRs expression

In view of the notion that high risk HPVs are known to efficiently evade immune recognition, we tested whether these viruses might subvert PRRs expression and function. We made use of high risk HPV-positive primary human KCs containing full length hrHPV genomes. We chose to use these cells, rather than cells transfected with plasmids comprising selected HPV genes, because they maintain episomal copies of the HPV genome and, upon culturing in organotypic raft cultures, display the entire differentiation-dependent HPV life cycle. Analysis of the TLR expression pattern of undifferentiated and differentiated monolayer cultures of HPV16-positive human foreskin KCs revealed essentially the same pattern as found for HPV-negative KCs, in that 6 TLRs are constitutively expressed (TLRs 1, 2, 3, 5, 6 and 10), 3 TLRs are not expressed in any differentiation stage (TLRs 4, 7 and 8), while TLR9 is induced upon KC differentiation. Thus, HPV16 does not directly affect TLR expression in KCs,
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nor the differentiation-induced expression of TLR9. Similar results were obtained for monolayer cultures of HPV16-positive vaginal KCs and HPV18-positive cervical KCs, while immunohistochemical staining of raft cultures derived from these KCs revealed that TLR9 is expressed at the protein level (unpublished). This suggests that our findings generally apply to high risk HPV types in the context of different types of stratified epithelia.

In spite of the fact that high risk HPVs do not appear to directly suppress TLR9 levels in KCs, HPV-associated defect in the TLR9 pathway was found through immunohistochemical analysis of normal and dysplastic genital epithelia for TLR9. In normal cervical epithelium, the staining intensity for TLR9 increased towards the most apical KC layers, in line with the notion that TLR9 expression is induced upon KC differentiation. However, TLR9 expression in the supra-basal and apical layers is gradually lost in dysplastic cervical lesions upon progression from stage CIN1 to stages CIN2 and CIN3. As is apparent from the immunohistochemical staining, the appearance of KCs with aberrant morphology in the supra-basal epithelial layers coincides with the loss of TLR9 expression. This result of HPV-induced dysplasia on TLR9 expression is vividly illustrated by the tissue section displayed in (Figure 1 in which a TLR9-positive normal region (left portion of section) and TLR9-negative CIN3 region (right portion of section) are shown side by side. Thus, HPVs do not directly block TLR9 expression in KCs, but rather prevent TLR9-expression in an indirect manner. Because chronic HPV infection interferes with KC differentiation, it also interferes with the differentiation-dependent induction of TLR9 expression.

Recent studies by others concerning expression and function of TLR9 in HPV-positive KCs and epithelia have resulted in divergent conclusions. Discrepancies between these studies focus on two issues: the direct impact of HPV E6/E7 on TLR9 expression and function, and the expression of TLR9 in normal versus dysplastic cervical epithelia. With respect to TLR9 expression and function in KCs, Anderson et al. showed that E6/E7 expression affected neither TLR9 expression and function, whereas Hasan et al. reported that E6/E7 expression in KCs resulted in loss of TLR9 expression and, therefore, function. These studies differ essentially from ours, in that they made use of human KCs displaying forced expression of the HPV16 E6 and E7 oncogenes as a result of gene transduction with E6/E7-specific expression vectors that are driven by strong viral promoter sequences. In contrast, we made use of KCs harboring episomal copies of entire HPV genomes, in which E6 and E7 levels are expected to be lower and the other HPV proteins are still present. Therefore, our results do not rule out that elevated E6/E7 levels, as expressed in the cell lines used by Hasan et al. and as
could be found in HPV-positive cancers, may have a direct suppressive effect on TLR9 expression. We cannot readily explain the complete absence of any impact of E6/E7 on the TLR9 pathway as reported by Andersen et al.\textsuperscript{10}. The second point of discussion concerns TLR9 expression in cervical epithelia in relation to KC differentiation and dysplasia. Whereas the study by Hasan and coworkers revealed the absence of TLR9 in immunohistochemical staining of HPV-positive cervical cancers\textsuperscript{12}, Lee et al. reported that such samples show greatly increased TLR9 staining\textsuperscript{20}. A peculiar feature of the data by Hasan et al.\textsuperscript{12} is that TLR9 staining is found in the basal layers of the normal epithelium. This observation, as well as the expression of TLR9 in tumor samples\textsuperscript{20}, would argue that TLR9 expression is inversely correlated with KC differentiation. Our data lead to the opposite conclusion. Importantly, our experiments concerning the relation between KC differentiation, TLR9 expression in KCs, and the presence of
HPV are fully consistent, in that (i) KC-differentiation results in induction of TLR9 expression in KC monolayer cultures, human epithelia and organotypic raft cultures, (ii) HPVs do not prevent the in vitro differentiation of KCs, nor the induction of TLR9 expression in either monolayer or raft cultures, while (iii) HPVs do interfere with both KC differentiation and TLR9-induction in the context of chronically infected human epithelia. As such, our study constitutes the first report that is likely to provide the correct picture for TLR9 in HPV infections.

3 Our unique data on HPV’s ability to dampen immunity at early phase of infection cycle

Based on the expression of several different PRRs by KC, infections with HPV should lead to the production of type I interferon as well as proinflammatory cytokines and chemokines. In order to understand how HPV infection manages to escape from the immune system, the interaction of HPV proteins with proteins belonging to immune signaling pathways has been studied. Our genome-wide approaches have identified that HPV upregulates about half of the genes differentially expressed between primary KCs and HPV-infected KCs, and downregulates the remainder in unstimulated keratinocytes. Among the upregulated genes we could identify that even at such an early phase of infection cycle high risk HPV upregulates the expression of cell cycle regulators. In addition, our transcription factor enrichment analyses identify many transcription factor binding sites, including motifs binding the important oncogene MYC that plays a critical role in hrHPV-induced cervical cancer\textsuperscript{21}. Our data thus suggest that high risk HPV even at the very early phase of the infection induces an oncogenic gene signature in infected keratinocytes and reprograms keratinocytes to cycle rapidly. The novelty of our data involves the genome-wide scale.

Among the genes downregulated by HPV infection were mainly genes involved in innate and adaptive immune responses of the host. Those genes can be broadly categorized in antimicrobials, inflammasomes, proinflammatory cytokines and chemokines, and antigen-presenting molecules. Thus, by upregulating cell cycle genes and by downregulating immune response genes, HPV creates favorable niche needed to establish persistent infection beneficial for the virus. Our data show that hrHPVs downregulate \textit{BAMBI}, a negative regulator of TGF-\(\beta\). The well known immunosuppressive roles of TGF-\(\beta\) requires further studies in that hrHPVs may suppress immunity by downregulating BAMBI. Similarly, genome-wide data obtained from our study should provide entry points for further studies to pinpoint the molecular mechanisms utilized by hrHPVs to deregulate host immunity.
So far, HPV has been shown to downregulate certain cytokines including type I IFN\textsuperscript{22}, MIP3A\textsuperscript{23} in candidate gene studies, but our unbiased genome-wide study has revealed downregulation of many pro-inflammatory cytokine and chemokines by HPV. Our gene network analyses revealed that \textit{IL1B} and \textit{IL6} are the most interconnected hubs downregulated by HPV. IL1B not only mediates inflammation but also links innate and adaptive immune responses. IL1B activates the release of other proinflammatory cytokines such as TNF and IL-6, and induces a TH17 bias in the cellular adaptive responses critical for the clearance of mucosal infection\textsuperscript{24}. As such, our data on the reduced production of IL1B in hrHPV-infected keratinocytes might be related to delayed induction of immune responses against HPV during the early phase of the infection cycle. Moreover, a recent study using HPV16 E6 and/or E7-immortalized keratinocytes shows strong downregulation of IL1B but not NALP3 by HPV E6. The same study further shows that pro- IL1B, precursor for mature IL1B, is degraded in a proteasome-dependent manner which is mediated via ubiquitin-ligase E6-AP and p53\textsuperscript{25}. Whether the reduced level of IL1B in HPV-transformed keratinocytes is also seen in cervical cancer patients needs to be further studied. IL1B is critical in linking innate and adaptive immunity and therefore reduced production of IL1B both at early HPV infection and in transformed cells might play important roles in deregulated innate and adaptive immunity in cervical cancer patients.

Nees et al.\textsuperscript{26} published a microarray-based study using primary human keratinocytes retrovirally transduced with HPV16 E6 or E7. Their study is essentially different from ours in that their system mimics the situation in transformed cells but not an early hrHPV infection. One of the other most notable differences with our study is that we used systems biology approaches to decipher complex networks and pathways in an unbiased fashion to identify a comprehensive effect of hrHPV in keratinocytes.

We\textsuperscript{27} analyzed our genome-wide data using approaches like KEGG database for pathway analyses, CORE_TF\textsuperscript{28} for over-represented transcription factor binding sites in promoters, Ingenuity Pathway Analyses (IPA) for gene network and pathway analyses which revealed many novel insights. As an example, HPV has been known to modulate ubiquitin-proteasome systems\textsuperscript{29} and we indeed observed significant enrichment of the protein ubiquitination pathway between HPV-infected and uninfected keratinocytes. Among the genes in the protein ubiquitination pathway, we identified UCHL1 as the most upregulated gene in HPV-infected keratinocytes with no known function in antiviral immunity. Our subsequent study has identified UCHL1 as a novel suppressor of innate immune signaling exploited by HPV to dampen innate immune responses of keratinocytes. In contrast to the viruses that actively inhibit host immune responses by viral encoded proteins, the genome size of HPV is relatively small. Therefore, HPVs
exploit the cellular machinery to evade host immune responses. Targeting of a cellular enzyme UCHL1 is a smart choice of the hrHPVs. By increasing the expression of UCHL1 that targets TRAF3, TRAF6, NEMO, and IκBα which are central to many PRR signaling pathways, hrHPVs simultaneously suppress many signaling routes leading to reduced activation of innate immunity of keratinocytes. However, our data show that UCHL1 downregulation alone is not sufficient to induce a strong, spontaneous innate immune response in hrHPV-infected keratinocytes. One reason for that is perhaps the inefficient knockdown of UCHL1 by RNAi (only about 20% reduction of $UCHL1$ mRNA after RNAi as detected by qRT-PCR, unpublished), thus keeping UCHL1 levels high enough to suppress immune responses of infected keratinocytes. Also, in addition to UCHL1, hrHPVs deregulate hundreds of other genes (see HPV signature genes) and it is possible that some of these genes play important roles in additional suppression of the immune responses of keratinocytes, signifying the need for further studies. Moreover, our data show that hrHPVs downregulate even the baseline levels of pro-inflammatory cytokines, chemokines and other molecules such as inflammasome components necessary for an effective innate immune response of keratinocytes. Our data show that RNAi inhibition of UCHL1 alone increases the baseline expression of proinflammatory cytokines in hrHPV-infected keratinocytes but not to a level as high as in uninfected keratinocytes, explaining why UCHL1 inhibition alone may not result in the induction of a spontaneous immune response and why super-stimulation with other immune activating molecules such as polyI:C would be desirable.

We found that high risk HPVs upregulate UCHL1 to dampen the immune responses of keratinocytes. Low risk HPVs (such as HPV6, and HPV11) are not associated with cancer but with genital warts. Given that it takes months to clear even low risk HPVs by host’s immunity, it would be exciting to examine if low risk HPVs also use UCHL1 to dampen the innate immunity of keratinocytes. Additionally, little is known about the normal physiological function of UCHL1. UCHL1 is highly expressed in neurons and UCHL1 polymorphisms have been associated with many neurodegerative diseases including Alzheimer’s disease and Parkinson’s disease. Chronic inflammation has been strongly linked to neurodegeneration and on the basis of our data of the novel function of UCHL1 in suppressing inflammation, it is tempting to speculate that chronic inflammation in the brain due to dysfunctional UCHL1 might lead to neuronal death and neurodegeneration. Further studies in this direction including the use of UCHL1 knockout mice are expected to yield exciting findings.

Our genome-wide study has revealed many other interesting downregulated genes, including the components of inflammasomes (NLRP2, PYCARD), while upregulation
was found of several antiviral response genes (TRIM5, IFIT2). This seemingly contradictory finding of upregulated antiviral genes and many downregulated proinflammatory genes needs to be studied in detail in order to understand how HPVs avoid immune response during early phase of infection cycle whilst most cases of HPV infection are cleared by the immune response at a later phase. Moreover, our data on the highly significant enrichment of genes belonging to the protein ubiquitination pathway between uninfected and hrHPV infected keratinocytes warrant further studies in order to understand how hrHPVs manipulate antigen presentation as ubiquitination controls antigen presentation at many stages. Our gene lists in enriched protein ubiquitination pathways show that hrHPV infection strongly downregulates the expression of genes encoding for antigen presenting molecules such as HLA-A, -B, and –C which needs to be studied further because in addition to affecting these molecules at gene level, one may envisage that similar to proteasomal degradation of pro-IL-1β by HPV16 E6 via the ubiquitin ligase E6-AP and p53, antigen-presenting molecules might also be targeted for degradation by hrHPV in a similar fashion. In support of this view, it is well known that other viruses prevent the presentation of viral peptides by selective degradation of MHC class I molecules. For example, the mK3 protein of mouse herpesvirus 68 binds to its primary binding partner TAP1/2 (transporter associated with antigen processing) and induces K48-linked polyubiquitination of MHC class I molecules resulting in their degradation. Moreover, the Kaposi’s sarcoma-associated virus (KSHV) proteins kK3 and kK5 and HIV protein Nef induce endocytosis of MHC class I molecules, leading to lysosomal degradation. It is known that hrHPVs inhibit antigen presentation to cytotoxic cells, however, the molecular mechanisms behind this are not well studied. It would be necessary to understand which E2 and E3 ubiquitin ligases as well as DUBs are exploited by HPV to inhibit antigen presentation pathways in order to better treat hrHPV infection and associated diseases.

4 Mimicking the in situ situation using keratinocyte differentiation-dependent HPV production, and interaction with immune cells

Our study is limited to the use of undifferentiated keratinocytes harboring episomal HPV DNA to mimic a genuine viral infection. It would be of great importance to closely mimic differentiation-dependent HPV production as well as the interaction of immune cells with cervical epithelium in situ by using organotypic epithelial raft culture comprising immune cells like Langerhans cells/dendritic cells (LCs/DCs), and use this material to perform a genome-wide study of HPV-positive keratinocytes during differentiation. Colonization of LCs/DCs into organotypic culture of HPV-transformed keratinocytes has been described to be minimal under basal condition. However, the infiltration of LCs/DCs in the in vitro formed pre-neoplastic epithelium is dramatically increased after the addition of inflammatory mediators such as GM-
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CSF to the culture\textsuperscript{34-36} suggesting that such immune cell-epithelial interactions mimic in situ situations at least in part. Moreover, very recently a model has been described to study the interaction between keratinocytes and T cells in a three-dimensional (3D) microenvironment that can recapitulate skin pathology due to migration of activated T cells into the dermis\textsuperscript{37}. Similar co-culture studies using the HPV-infected keratinocytes and various immune cells are needed in order to better understand how modulation of innate immune responses in KC affect their interaction with immune cells and vice versa.

5 How would chronic HPV infection lead to dysfunctional adaptive immunity in patients with neoplasia and cervical cancer based on the data presented in this thesis?

Our studies reveal that many of the molecules involved in innate immunity are downregulated by HPVs. For instance, HPV-infected keratinocytes strongly downregulate the secretion of proinflammatory cytokines and chemokines. As there are fewer proinflammatory signals, the other cells of the immune system including Langerhans cells, DCs, and NK cells will not be attracted to the site of HPV infection. Thus HPV infection remains undetected by the immune system. Although hrHPVs suppress the immune responses at the early phase of the infection cycle by employing UCHL1, activation of immune responses is observed in a later phase of cervical cancer (grade III and cancer)\textsuperscript{18} and loss of UCHL1 might play a crucial role in this later process. UCHL1 has been shown to function as a tumor suppressor in several types of human cancers that is inactivated by promoter methylation or gene deletion\textsuperscript{38}. The activity of UCHL1 has been shown to be lower in a significant proportion of cervical cancers compared to the adjacent normal tissues and UCHL1 expression is undetectable in almost all cervical cancer cell lines\textsuperscript{39} suggesting that UCHL1 expression is lost during cervical tumorigenesis. Chronic inflammation has been linked to the development of cancer\textsuperscript{40} and loss of UCHL1 expression might play roles in this process. At the early phase of the HPV infection cycle when viruses are produced, the viruses need to suppress immune responses in order to establish chronic infection. However, after chronic infection has been established and when the HPV oncogenes E6 and E7 integrate into the cellular genome, suppression of immune responses is no longer important as infectious viruses are not produced. Rather, the cells need to survive and proliferate which is mediated by the activation of NF-κβ and other pro-survival pathways\textsuperscript{26}. UCHL1 being a strong suppressor of various PRR pathways that lead to reduced activation of NF-κβ, it is tempting to speculate that UCHL1 inactivation would lead to chronic activation of NF-κβ, ultimately leading to the development of cervical cancer. Similar to UCHL1, CYLD is a DUB that negatively regulates the activation of NF-κβ. A recent study
Chapter 6 shows that HPV E6 mediates proteasomal degradation of CYLD in cervical cancer cell lines, thereby allowing hypoxia-induced NF-κβ activation and tumorigenesis. Since UCHL1 expression is undetectable in almost all cervical cancer cell lines, further studies examining DUB expression in the context of NF-κβ activation in cervical cancer are necessary.

6 What might be the reasons why HPV infection cannot be cleared by a minority of the subjects leading to the development of cervical cancer?

Although it is clear that the innate immune response to HPV is suppressed, thereby delaying the induction of protective immune responses, at the end of the day the majority of the HPV infections are controlled, associated with detectable HPV-specific T cell responses. So what might be the problem with the immune responses of the individuals who display progressive infections and finally develop cervical cancer? The subsequent establishment of persistent infection may have been influenced by a combination of genetic and environmental factors. In the case of genetic factors related to innate or adaptive immune system, the ‘defects’ are expected to be subtle, because subjects with HPV infections generally do not show increased susceptibility to other opportunistic pathogens. The antigen presenting machinery (APM) plays a crucial role in immune recognition of virally infected cells and cancer cells and single nucleotide polymorphisms (SNPs) at several loci in the APM genes have identified the major alleles at the LMP7 and TAP2 loci and the minor allele at the ERAP1 locus to be significantly associated with increased risk of cervical carcinoma. LMP7, TAP2 and ERAP1 are critical components in the HLA Class I (including HLA-A, -B, and -C) antigen presentation machinery. LMP7 processes intracellular proteins into peptides which are then transported from the cytoplasm to the endoplasmic reticulum by TAP2. Transported peptides undergo length-specific trimming by ERAP1 before being loaded onto HLA-A, -B, and -C. Our data that hrHPVs strongly downregulate HLA-A, -B, and -C at early infection cycle and the data of Metha et al., that certain polymorphisms in HLA-A, -B, and -C are associated with increased risk of cervical cancer indicate that these antigen presenting molecules play critical roles in immunity against hrHPV infection and in cervical cancer. Moreover, genome-wide association studies have recently identified SNPs within the MHC regions including various SNPs (i) At HLA-DPA1 and HLA-DPB1/2, (ii) A gene (MICA), adjacent to the MHC class I polypeptide-related sequence, and (iii) Gene(s) between HLA-DRB1 and HLA-DQA1 that affect susceptibility to cervical cancer in situ probably by causing impaired immune activation. Interestingly, hrHPV-specific T cells infiltrating cervical cancer and lymph nodes are predominantly restricted via HLA-DQ and –DP, sustaining the notion that
these genes/molecules play an important role in immunity to hrHPV positive tumors. Therefore, it would be interesting to study polymorphisms of the genes strongly affected by HPV infection identified by our genome-wide analysis including IFNα, IFNβ, IL1A, IL6, and CCL5. Moreover, the proteins in the PRR signaling pathways that are targeted by UCHL1 namely TRAF3, TRAF6 and NEMO as well as UCHL1 itself could be the prime molecules to study their polymorphisms. Additionally, polymorphisms of all the members of the PRR pathways including the receptors TLRs, NLRs, RLRs, as well as the downstream molecules MYD88, IRAK1, IRAK4, TBK1, IKKα, IKKβ, IκB, p50, p65, IRF3, IRF7 might be important suspects. Certain polymorphisms of these crucial molecules may contribute to dysfunctional innate and adaptive immune responses and therefore HPV persistence and ultimately the development of cervical cancer.

7 UCHL1 as a therapeutic target for chronic HPV infection

Imiquimod is an immune-system activator that induces local inflammation when topically applied. It is used in the clinic for the treatment of vulvar intraepithelial neoplasia (VIN). Similar approaches are also successfully tested to treat the HPV-infected region of cervix. These studies indicate that the induction of local inflammation is essential to control HPV infections, however, the response rate in these studies still requires improvement. Therefore, other approaches of immune-system activation to eradicate chronic hrHPV infection are warranted. Targeting the ubiquitin proteasome system (UPS) offers a potential solution as suggested by the treatment of multiple myeloma. This identifies UCHL1 as a potential therapeutic target to treat chronic hrHPV infection. UCHL1 is absent in normal keratinocytes, however, its expression is strongly induced upon hrHPV infection. Therefore, blocking of UCHL1 function might not pose a threat to the normal physiology of cervical epithelia. In combination with immunostimulatory agent(s), blocking of UCHL1 function by small molecules, anti-sense oligonucleotides or monoclonal anti-UCHL1 antibodies during the chronic phase of HPV infection would lead to the activation of innate immune responses of HPV-infected keratinocytes and subsequently the activation of adaptive immunity which would lead to the clearance of persistent HPV infection. Various small molecules including isatin O-acyl oxime are currently known that interfere with the activity of UCHL1. Anti-sense oligonucleotides including RNAi, shRNA, miRNA are designed to base pair to specific nucleotide sequences, and thus, they potentially offer a lower risk for off-target effects than do small-molecule drugs. Recently, the FDA has approved Kynamro ( mipomersen sodium), a novel antisense oligonucleotide inhibitor for the treatment of inherited cholesterol disorder underscoring the importance of anti-sense UCHL1 therapy against chronic hrHPV infection. However, anti-sense therapy
has disadvantages of poor intracellular uptake, and high toxicity. Because of the various advantages of monoclonal antibody mediated therapy including target specificity and generally well tolerated with mild side effects, monoclonal-anti-UCHL1 antibodies would be desirable. Antibodies are viewed as too large to access intracellular locations. Therefore, antibody therapy has traditionally targeted extracellular or secreted proteins expressed by cells. However, recent study by Guo et al., showed that exogenously provided antibodies or vaccine-induced antibodies against intracellular proteins (namely EGFP and PRL-3, or the polyoma middle T oncoprotein) delayed the growth of a variety of tumors that expressed these intracellular proteins suggesting that intracellular proteins can even be targeted by therapeutic antibodies. This study suggests the possibility that monoclonal anti-UCHL1 antibodies may constitute a viable option for the treatment of chronic hrHPV infection. However, blocking of UCHL1 function alone might not trigger a full-blown spontaneous immune response against hrHPV (our data as discussed above). Therefore, an additional trigger (like polyI:C as our data show) to stimulate the immune system might be desirable. Our data show that keratinocytes secrete inflammatory cytokines upon treatment of polyI:C or flagellin, in line with the PRR expression (TLR3/RIG-I and TLR5 respectively) of keratinocytes indicating that those TLR-agonist could potentially be used to further activate the immune system in addition to the UCHL1 blockade. In contrast, TLR7 and TLR8 are not expressed in keratinocytes. Therefore, the TLR7/8 agonist imiquimod will not directly activate the innate immunity of keratinocytes, however, imiquimod treatment may activate other epithelial resident immune cells including T cells, DCs, pDCs, Langerhan’s cells as suggested by Terlou et al.,. The proinflammatory milieu produced by resident DCs, pDCs, and Langerhan’s cell following imiquimod treatment may therefore indirectly activate the HPV-infected keratinocytes enabling the presentation of hrHPV antigen to T cells implying that imiquimod treatment on top of UCHL1 blockade may increase the effectiveness in clearing chronic HPV infection.

8 Cancer promoting inflammation through chemokine receptors

Chronic inflammation has been associated with cancer, and chemokines play a strong role in such inflammation. Our study has revealed that while HPV downregulates molecules involved in innate immunity of keratinocytes, surprisingly, upregulation occurs of genes involved in cancer promoting inflammation, even at the early phase of HPV infection cycle. Our microarray study using HPV-infected and uninfected keratinocytes revealed that chemokine receptor CXCR7 is highly upregulated in infected keratinocytes. The role of CXCR7 upregulation in hrHPV-infected keratinocytes needs to be studied further. However, based on the various signaling pathways activated upon ligand binding to CXCR7 i.e., PKC (Protein Kinase C), Akt, it maybe speculated that
CXCR7 upregulation in hrHPV-infected keratinocytes would lead to increased survival and proliferation of keratinocytes, fitting with the HPV infection cycle. CXCR7 expression remains high in tumor cells of cervical cancer patients. Our further analyses show that CXCR7 expression in tumor cells is associated with tumor size, lymph node metastasis, and disease-free survival in cervical cancer patients in agreement with the known function of CXCR7 in cell proliferation, and trans-endothelial migration/metastasis\textsuperscript{54}. Our patient-derived data suggesting the existence of the CXCR7-EGFR co-receptors are in line with the cancer cell derived cellular and biochemical data presented by Singh\textsuperscript{55}. By coupling/colocalizing with EGFR, CXCR7 may be responsible for increased tumor growth and metastasis, but the various pathways downstream of EGFR (the RAS-BRAF pathways) and the cross-talk happening through dimerization with other receptors (HER-2, HER-3, and HER-4) need to be studied.

9 Regulation of T-cell mediated immunity in cervical cancer

PD1-PDL1 interaction has been linked to dysfunctional adaptive immunity due to T cell exhaustion. HPV-specific T cells are generally undetectable or impaired in patients with cervical neoplasia and cervical cancer\textsuperscript{18, 56, 57}. We were the first to study PD-L1 expression and possible function in cervical cancer patients. Our data, in contrast to our expectations as published for many other cancers, show that the minority of cervical cancer cells express PD-L1. Very recently, it has been described that the prevalence of cell surface staining and staining intensity in the paraffin-embedded section is slightly less than in the frozen specimen\textsuperscript{58} using the PD-L1 antibody (5H1 clone) that we also used for the IHC of our paraffin-embedded cervical cancer patient specimens suggesting that there might be an underestimation of the true PD-L1 expression in our study due to technical problems. Repetition of our study may, therefore, be needed to sustain or refute our notion that the PD-L1 expression by cancer cells does not play such a role in cervical carcinoma. Furthermore, we found that about 50% of the infiltrating T cells express PD1. Recently, it has been shown that the levels of PD1-positive tumor-infiltrating T cells are positively correlated with a favorable clinical outcome in HPV-associated head and neck cancer\textsuperscript{59}. Further studies on those PD-1-positive T cells show that they express T cell activation markers and about 50% of these cells do not express additional T cell inhibitory receptor TIM-3. Therefore, in order to conclusively show that the PD1 expressing T cells in cervical cancer are functionally impaired, the presence of additional T cell inhibitory receptors like TIM-3, LAG-3, CTLA-4 should also be studied\textsuperscript{60, 61} to conclusively phenotype the nature of PD-1-positive tumor-infiltrating T cells we observed in cervical cancer patients.
Assuming that PD-L1 expression indeed is scarcely expressed by cervical cancer cells, then what are the other sources of PD-L1 in the cervical cancer microenvironment? In order for intratumoral PD1+ T cells to become functionally blocked, receptor-ligand interaction is needed, i.e., PD1+ T cells should bind PD-L1. One source could be the co-infiltrating immune cells. Similar to the situation in lung cancer, immature DC in tumor microenvironment could express high levels of PD-L1. DCs cross-presenting tumor antigen (e.g., E6 and E7 of HPV) in the tumor microenvironment may express PD-L1 which may impair the function of responding PD1+tumor-specific T cells. Additionally, high levels of PD-L1 expression on monocytes and macrophages have been shown to effectively suppress tumor-specific T cell immunity and to contribute to the growth of human hepatocellular carcinoma cells in vivo. Notably, in a cell culture system, human cervical cancer cells either hampered monocyte to dendritic cell differentiation or skewed their differentiation toward M2-like macrophages which express high levels of PD-L1. This is substantiated by HPV-associated head and neck squamous cell carcinomas (HPV-HNSCC) displaying high infiltration with PD-L1 expressing tumor-associated macrophages which were suggested to inhibit the function of PD1 expressing tumor-infiltrating T cells. A recent study shows that M2 macrophages are often present in high numbers in patients with cervical cancer. The M2 macrophages were not directly associated with the clinical outcome of the patients, suggesting that they would not play a role in cancer progression, however, their influence in the context of PD-1 expressing T cells has not been examined. Furthermore, if such tumor-infiltrating M2 macrophages expressing additional inhibitory co-receptors that could also terminate T-cell responses need to be studied.

PD-1 and PD-L1 blockade with monoclonal antibodies has emerged as a very promising and successful treatment approach for patients with metastatic melanoma, non-small cell lung carcinoma, and metastatic colorectal cancer. What about the therapeutic potentials of PD-1 and/or PD-L1 blockade in cervical cancer patients? As described above, a number of studies need to be performed to provide a more definitive answer to this question, including the use of the specialized immunohistochemistry procedures for the detection of PD-L1 suggested by the experts in this field and studies on the impact of PD-L1 expressing myeloid cells in the microenvironment. Furthermore, one could also think about other therapeutic antibody options but this requires studies on the co-expression of other inhibitory receptors such as TIM-3, LAG-3, and CTLA-4.
References


