DEREGULATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES IN HUMAN PAPILLOMAVIRUS INFECTION AND CANCER

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To my parents, Henrike (wife), Norah and Ruben (children)

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Abbreviations used frequently

HPV, human papilloma viruses; hrHPV, high-risk human papilloma viruses; KCs, keratinocytes; PRRs, pattern recognition receptors; TLRs, Toll-like receptors; NLRs, Nod-like receptors; RLRs, RIG-I-like receptors; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TRAFs, TNF-receptor-associated factors; NEMO, NF-κB essential modulator; DUBs, deubiquitinating enzymes; UCHL1, ubiquitin carboxyl-terminal hydrolase L1; APCs, antigen-presenting cells; Tregs, regulatory T cells; PD-1, programmed death 1; B7-H1/PD-L1, B7 homolog 1/ programmed cell death 1 ligand 1; B7-DC/PD-L2, B7 homolog DC/ programmed cell death 1 ligand 2; CXCR7, C-X-C chemokine receptor type 7; EGFR, epidermal growth factor receptor.

Chapter 1

Introduction

1 Human papilloma viruses

Human papilloma viruses (HPV) are non-enveloped double stranded DNA viruses (7-8 kb) that infect human skin and mucosa and are the causative agents of mostly benign proliferative lesions such as common (genital) warts [1]. However, persistent infection with 'high-risk' HPV subtypes is associated with the development of anogenital malignancies such as cervical, vulvar, penile and anal cancer, and also a growing subset of oropharyngeal cancers [2, 3]. The association is the strongest for cervical cancer as illustrated by the finding that HPV DNA can be detected in over 99% of cervical cancers [4]. Notably, cervical cancer is the second most common cancer in women worldwide, with an estimated death toll of almost 300,000 women annually, mostly in developing countries [5].

More than 180 types of HPV are known and 15 are thought to be high-risk and tumorigenic [6]. HPV16 is the most common high-risk type, and responsible for about half of all tumors; HPV18 accounts for another 10-15%; and HPV types 31, 33, 45, 52, and 58 account for an estimated 2-5% each [7].

1.1 Squamous epithelia

The surface of the human body that is exposed to the outside milieu is largely covered by stratified squamous epithelia, which are built up of multiple layers of keratinocytes (KCs) to create a robust physical barrier. Examples of such epithelia are the skin and the lining of the oropharyngeal and the lower female reproductive tracts. Tightly regulated division of the undifferentiated KCs in the basal layers results in the continuous production of new cells that gradually differentiate as they move to the upper layers in the epithelium, where they can serve as replacement for the cells that are lost from the epithelial surface due to wear and tear. The apical layers are not only exposed to physical stress, but also to a great variety of infectious pathogens, including bacteria and viruses. Similarly, the cells in the basal layers can be targeted by pathogens through wounds and micro-abrasions.

1.2 The viral life cycle

HPV infects the keratinocytes in the basal layer of the epidermis and mucosal epithelium, and the viral life cycle is tightly regulated through the differentiation program of keratinocytes [3]. Following infection and uncoating, the virus genome is maintained as episomes at a low copy number in the basal cells of the epithelium, where proliferation-inducing early genes (including E6 and E7) are expressed, resulting in lateral expansion of the infected cells. Later, the suprabasal layers of epithelium support viral replication where hundreds to thousands HPV genomes are present within a single cell. The L1 and

L2 capsid proteins are expressed in the most superficial layers of the epithelium where viral assembly takes place, and finally new infectious viral particles are released [1, 3].

1.3 Malignant transformation

For high-risk HPV infections, E6 and E7 effectively block the negative regulators of the cell cycle, whereby the cells remain active in cell cycle progression with the cessation of differentiation and apoptosis. As such, the infected cells acquire genomic instability and genetic alterations, ultimately driving malignant transformation of an infected cell into an invasive cancer cell.

E6 and E7 start oncogenesis by inactivating tumor suppressors. The tumor suppressor protein E6 targets TP53 for degradation via the ubiquitin proteasome pathway, preventing apoptosis and enabling potentially transformed cells to replicate [8]. The tumor suppressor protein E7 contributes to oncogenesis through its interaction with the retinoblastoma tumor suppressor family members RB1, RBL1 and RBL2 and targets them for degradation [9].

2 Innate Immunity

The mammalian innate immune system provides a first line of defense against microbial attack through antimicrobial factors, phagocytosis and the induction of inflammation. Mucus covers the internal surface of the anogenital tracts and functions to trap infectious microorganisms and pollutants. Mucus contains mucins and various other microbicidal molecules, including antimicrobial peptides (calprotectin, lysozyme, lactoferrin), secretory leukoprotease inhibitor, and human β-defensins), immunoglobulins, and complement factors that directly bind to and kill microorganisms before they reach the host epithelial cell layer.

Invading viruses and microbes contain pathogen-associated molecular patterns (PAMPs) that are recognized by the host's pattern recognition receptors (PRRs). Two main classes of PRRs have been described in mammalian cells: 1) membrane-bound receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and 2) cytoplasmic sensors, including NOD-like receptors (NLRs), pyrin and HIN domain-containing (PYHIN) family members, RIG-I-like receptors (RLRs) and an increasing range of cytosolic nucleic acid sensors [10]. All of these receptors activate conserved signaling cascades that lead to activation of NF-κB via the canonical route, while RLRs and some TLRs activate interferon regulatory factors (IRFs) that together with NF-κB induce the production of type I interferons (IFN) and other effector molecules. Other PRRs initiate the assembly of cytoplasmic signaling complexes, termed inflammasomes,

which activate inflammatory caspases and cause maturation and secretion of IL-1β and IL-8. Each member of the PRR family recognizes distinct PAMPs. TLRs recognize a diverse array of PAMPs including bacterial lipoproteins, lipopolysaccharide (LPS), flagellin, peptidoglycan, nucleic acids as well as viral glycoproteins and nucleic acids, such as double-stranded RNA (dsRNA), uncapped single-stranded RNA (ssRNA) and viral DNA. NLRs recognize peptidoglycan fragments and RNA while RLRs comprising retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and LGP2 (also known as DHX58) detect several different ssRNA and dsRNA viruses [10].

2.1 Expression of pattern recognition receptors in keratinocytes

KCs are equipped with sensors for pathogens, including TLRs, protein kinase R (EIF2AK2), and the RNA helicases RIG-I (DDX58) and MDA5 (IFIH1) which enable them to generate pro-inflammatory and anti-viral signals in response to PAMPs. Of these receptors, TLR1, 2, 4, 5, 6 and 10 are expressed at the cell surface and specialized in the detection of macromolecules that constitute the building blocks of pathogenic micro-organisms, while TLR3, 7, 8 and 9 protrude into the lumen of intracellular vesicles and detect nucleic acids of foreign origin [11]. RIG-I and MDA5 are cytosolic sensors [12]. The vast majority of published studies have shown that KCs express TLR 1, 2, 3, 5, 6, but lack TLR 7 and 8, while different conclusions were reached with respect to the expression of TLR4 and 9 in these cells [13-19]. In KCs, activation of these PRRs leads to direct NF-kappa-B activation and results in the upregulation of pro-inflammatory cytokines including IL-8, CCL2, CCL20, CCL27, and/or activation of type I interferon (IFN) response genes including transcription factors IRF3 and IRF7 regulating the production of antiviral cytokines [16].

2.2 Ubiquitins regulate cell signaling

Post-translational modification of proteins by ubiquitination regulates many cellular processes including the generation of innate and adaptive immune responses to pathogens[20, 21]. Ubiquitin is a highly-conserved 76-amino-acid polypeptide that can be covalently attached to cellular proteins through an enzymatic cascade involving three classes of enzyme termed E1, E2 and E3. E1 enzyme activates ubiquitin. Activated ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme. The E2 enzyme-ubiquitin complex interacts with an E3 ubiquitin ligase that facilitates transfer of the ubiquitin to a lysine (K) residue on substrate protein. Ubiquitination can be reversed by deubiquitinating enzymes (DUBs). In humans, there are two E1 enzymes, about 50 E2 enzymes and 600 E3 enzymes and about 100 DUBs.

A protein can be modified on one lysine residue with a single ubiquitin (monoubiquitination) or with a chain of ubiquitin (polyubiquitination). Lysine 48 (K48)-linked polyubiquitination usually targets protein for proteosomal degradation, whereas K63-linked polyubiquitination is involved in activating proteins in signal transduction cascades (Figure 1).

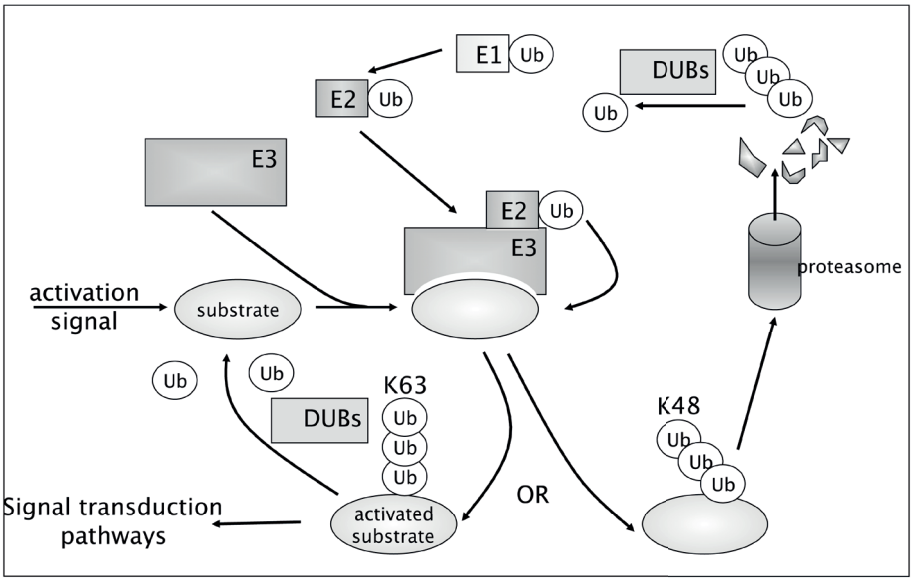

Figure 1 | The Ubiquitin-Proteasome System (UPS). First, ubiquitin activating enzyme, E1, forms a thioester linkage with the C-terminal glycine residue of ubiquitin (Ub) in an ATP-dependent manner. Ub is then transferred to ubiquitin conjugating enzyme, E2. Finally, E2 enzyme binds to ubiquitin ligase enzyme, E3, and the complex mediates isopeptide linkage formation between carboxy terminal glycine residue of Ub and lysine ε-amino group of the substrate. Repetition of this catalytic cycle leads to polyubiquitination of the substrate. Ub primarily binds to the substrate either through its N-terminal or other internal lysine residues (K6, K11, K27, K29, K33, K48 and K63). While K48 polyubiquitination marks the protein for proteasomal degradation, K63 polyubiquitination activates the protein leading to the activation of signaling pathways. Deubiquitinating enzymes (DUBs) remove Ub from polyubiquitinated proteins and recycle Ub during protein degradation (Adapted from S.H. van der Burg, unpublished).

However, pathogens have evolved many ways to exploit the ubiquitination system of the hosts. A common evasion strategy for viruses is to target key immune proteins for degradation. By degrading host's adaptor and signaling molecules, viruses disable many immune response pathways including the production of interferons and other innate host defense mechanisms. Additionally, viruses inhibit the ligation of ubiquitin or remove ubiquitin from host cell proteins to favor propagation and pathogenesis

of viruses. For instance, the NS5 proteins of dengue virus inhibits IFN signaling by selective ligation of K48-linked polyubiquitin chains in STAT2, thus promoting the degradation of STAT2, an essential component of ISGF3 complex required for ISG induction [22, 23]. Moreover, Epstein-Barr virus encoded BPLF1 protein acts as a deubiquitinase and removes ubiquitin from TRAF6 to inhibit NF-κB signaling during lytic infection resulting in enhanced lytic replication of the virus [24].

2.3 Regulation of innate immunity by ubiquitination

TLR pathways use two main adaptor proteins: myeloid differentiation primary response protein 88 (MYD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). MYD88-dependent pathways are used by all TLRs except TLR3 while TRIF-dependent pathways transmit signals from TLR3 and TLR4. Downstream of both MYD88 and TRIF-dependent pathways, ubiquitination plays critical roles in the activation of NF-κB and the mitogen-activated protein kinase (MAPK) signaling cascades. Following activation of MYD88-dependent pathways, MYD88 recruits kinases of the IL-1 receptor-associated kinase (IRAK) family, which then recruit TNF-receptor-associated factor 6 (TRAF6), a ubiquitin E3 ligase. Together with a ubiquitin E2 complex containing UBC13 and UEV1A, TRAF6 catalyze the synthesis of K63-linked polyubiquitin chains (Figure 3). These polyubiquitin chains bind to TAK1-binding protein 2 (TAB2) and TAB3 leading to the activation of the TGF-β-activated kinase 1 (TAK1) and the downstream MAPK cascade. K63-linked polyubiquitin also bind to the NF-κB essential modulator (NEMO), a regulatory subunit of the IκB kinase (IKK) which contains the IKKα and IKKβ catalytic subunits. Binding of K63-linked polyubiquitin chains to both the NEMO and TAK1 complexes facilitates the phosphorylation of IKKβ by TAK1, leading to the activation of IKK. IKK phosphorylates NF-κB inhibitor (IκB) proteins which are then recognized by the SCF-βTrCP ubiquitin E3 ligase complex targeting the IκB for K48-linked polyubiquitination and subsequent degradation by the proteasome. This allows NF-κB to enter the nucleus to turn on the target genes [20] (Figure 2).

In TRIF-dependent pathways downstream of TLR3, receptor-interacting protein 1 (RIP1) undergoes K63-linked polyubiquitination by ubiquitin E3 ligases such as TRAF6. RIP1 polyubiquitination recruits TAK1 and NEMO, leading to the activation of NF-κB. TRIF also recruits another ubiquitin E3 ligase, TRAF3, which activates the kinases TBK1 and IKKε, leading to interferon responsive factor 3 (IRF3) phosphorylation and type I IFN production. Additionally in IFN-induction pathway, TRAF3 and TRAF6 are recruited, TRAF3 undergoes K63-linked polyubiquitination leading to IFN induction by unknown mechanisms [20].

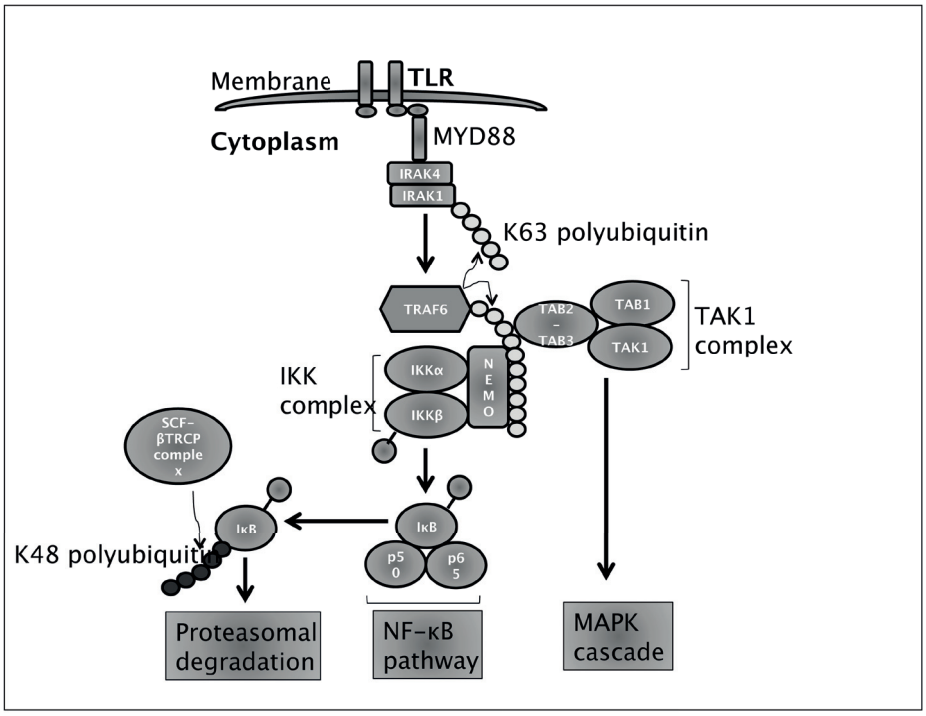

Figure 2 | Ubiquitination-mediated signaling in TLR pathways. TLR stimulation recruits MYD88, IRAK4, and IRAK1 further activating TRAF6. Activated TRAF6 synthesizes K63 polyubiquitin chains which bind either to TAK1 complex leading to the activation of MAPK pathway or to IKK complex through K63 polyubiquitination of NEMO resulting in the activation of IKK complex. Activation of both the TAK1 and IKK complexes facilitate the phosphorylation of IKKβ inducing further phosporylation of NF-ϰB inhibitor (IϰB) proteins. SCF-βTRCP ubiquitin E3 ligase complex then targets IϰB proteins for proteasomal degradation through ligation of K48 polyubiquitin chains, thus allowing NF-ϰB to enter the nucleus to turn on target genes involved in immune responses (Adapted from Jiang and Chen, Nature Reviews Immunology 12, 35-48 (2012)[20].

Ubiquitination plays crucial roles too in the RIG-I and melanoma differentiation-associated gene 5 (MDA5)-mediated pathways that sense viral RNA [21, 25]. Viral RNA binds RIG-I and induces a conformational changes that exposes the N-terminal CARD domains of RIG-I which binds the unanchored K63 polyubiquitin chains synthesized by TRIM25 and Riplet ubiquitin E3 ligases. RIG-I then interacts with and activates the mitochondrial membrane protein MAVS, further recruiting K63-linked polyubiquitinated TRAF6, TRAF3, TRAF2/5, and cIAP1/2 ultimately activating IKK and TAK1 complexes [21, 25] similar to TLR signaling as described above.

2.4 Regulation of innate immunity by deubiquitination

DUBs are proteases that cleave ubiquitin from target proteins and therefore oppose the function of ubiquitin E3 ligase. Consistent with a key role of ubiquitination in activating immune signaling cascades, several DUBs have been shown to negatively regulate immune responses. A20 and CYLD are two best known DUBs that inhibit PRR pathways [20].

Overexpression of A20 inhibits NF-κB activation in response to TNF or IL-1 stimulation. Mice lacking A20 die shortly after birth due to multi-organ tissue inflammation and cachexia [26] due to uncontrolled activation of NF-κB. Mechanistically, A20 inhibits NF-κB via its DUB domain by removing or inhibiting the K63-linked polyubiquitination on key NF-κB signaling molecules such as TRAF6, RIP1 and RIP2, thereby, inhibiting proinflammatory outcomes of PRR pathways. Additionally, A20 contains one of the C-terminal zinc finger domains (ZnF4) that harbor intrinsic E3 ligase activity selectively conjugating K48-linked polyubiquitin chains onto target molecules and cause their degradation. Therefore, A20 is a novel ubiquitin-editing enzyme with both DUB and E3 ubiquitin ligase activity [20].

Additionally, CYLD is a tumor suppressor, and loss of CYLD function has been linked to several types of skin tumor. CYLD contains a ubiquitin protease domain which specifically cleaves K63-linked polyubiquitin chains, thus functioning as a suppressor of NF-κB signaling. CYLD is a NEMO-interacting protein that inhibits IKK and NF-κB by removing K63-linked polyubiquitin chains from TRAF2, TRAF6, and NEMO. Other CYLD substrates important for NF-κB regulation include TAK1, Bcl3, and RIP1 [27]. HPV E6 has been shown to degrade CYLD in cervical cancer cell lines [28] possibly contributing to the development of cancer.

2.5 HPV and innate immunity

Anogenital HPV infections are very common and the cumulative lifetime incidence of infections is estimated to be as high as 80-85% [29]. However, most of the lesions are cleared and low-grade CIN lesion often regress spontaneously indicating that in the majority of individuals the immune system succeeds in controlling the viral infection before malignant disease develops. The prevalence of persistent HPV infections and HPV-positive lesions is greatly increased in immunosuppressed subjects, such as transplant recipients and HIV-positive patients, indirectly indicating that the immune systems plays a major role in controlling the HPV infections [30-33].

In healthy individuals, the duration of a transient anogenital detectable HPV infection, before it is controlled and viral DNA becomes undetectable, ranges from 7-14 months [34, 35]. This indicates that active viral infections are capable of persisting in the host for quite some time. This lag time suggests that HPV is able to evade and/or interfere with the innate and adaptive immune defenses [36]. Effective avoidance of immunity might be related to the characteristic infectious cycle of HPV. HPV life cycle is non-lytic and therefore does not elicit a strong proinflammatory signals to attract and activate the APCs as would be generated by dying cells of the host. Additionally, there is no blood-borne or viremic phase of the life cycle. This suggests that HPV-infected KCs need to sense the infection in order to activate an immune response. [37]. Additionally, HPV does not infect DCs, nor does it express its protein in DCs, therefore, DC need to cross-present the HPV early antigens derived from HPV-infected KCs in order to mount immunity against HPV [38]. Thus priming of antiviral immunity depends on highly specialized APCs, such as the Langerhans cells (LCs) that can access HPV proteins in the epidermis. Clinical observations show that the number of LCs is significantly reduced at sites of HPV infected premalignant lesions [39, 40]. Adhesion molecules, such as E-cadherins, are necessary to mediate contact between LCs and keratinocytes but HPVs reduce the expression of E-cadherins on keratinocytes cell surface [41], suggesting that innate immune signaling, required for the recruitment of APC, is altered by HPV.

HPV oncoproteins have been shown to downregulate the expression of type I interferons and pro-inflammatory cytokines of keratinocytes [42]. For instance, retrovirally transfected HPV16 E6/E7 in keratinocytes show reduced production of MIP-3α, the most potent chemotactic agent for LC precursors, which results in reduced migration of LCs to the site of HPV infection t and helps HPV to persist [43].

The type I interferons (IFN-α, and IFN-β) have antiviral, anti-proliferative, anti-angiogenic, and immunostimulatory properties and act as a bridge between innate and adaptive immunity [44]. Both in vitro and in vivo data suggest that HPV has evolved mechanisms to avoid the effects of type I IFN. IFN-α does not effectively inhibit transcription of E6/E7 RNA in several cervical epithelial cell lines immortalized by recombinant HPV16, HPV18, and HPV33 DNA [45]. Furthermore, pre-malignant lesions from patients non-responsive to treatment with IFN-α have higher levels of E7 mRNA compared to patients that respond to treatment, suggesting that HPV E7 may inhibit IFN signaling [46]. E7 binds to IRF-9 and prevents the translocation of IRF-9 to the nucleus, thus inhibiting IFN-α-mediated signal transduction by preventing the formation of the ISGF-3 transcription complex that functions by binding to the interferon-specific response element (ISRE) in the nucleus [47, 48]. Moreover, E7 binds to IRF-1 and inhibits

the activation of IFN-β through recruitment of histone deacetylase to the promoter, thereby preventing transcriptional activation [49]. Additionally, HPV18 E7 inhibits the transactivating function of IRF-1 resulting in reduced expression of IRF-1 target genes, such as the TAP1, IFN-β, and MCP-1 genes [50]. The HPV E6 protein also targets the interferon pathway. By binding to IRF-3 and preventing its transcriptional activation, E6 prevents transcription of IFN-α mRNA [51]. E6 also binds to TYK2 which prevents its binding to the cytoplasmic region of IFN-α receptor 1, and inhibits phosphorylation of TYK2, STAT1, and STAT2, thereby impairing the JAK-STAT signaling pathway resulting in reduced secretion of IFNs [52]. More recently, microarray analysis showed that HPV16 E6 [53] and HPV31 downregulates multiple IFN-responsive genes. E6 decreased expression of IFN-α and IFN-β, downregulates nuclear STAT-1 protein, and decreased binding of STAT-1 to the ISRE [53]. In addition, HPV16 E6 has been shown to degrade pro-IL-1β in a proteasome dependent manner which is mediated via ubiquitin ligase E6-AP and p53 [55].

2.6 Our questions

So far studies addressing HPV-mediated immune evasion have used retrovirally transduced HPV E6 and/or E7, mimicking the situation of KC transformation by HPV or have even used HPV-transformed KC or cervical cancer cell lines. While these studies are of interest with respect to the effects of HPV proteins on the immune response during malignant transformation, they are less likely to reflect how HPV escapes during infection because during cellular transformation the HPV E6 and E7 genes are integrated into the cellular genome and the production of infectious viruses is stopped. Furthermore, the expression of the different HPV early proteins (E1, E2, E4, E5, E6 and E7) is different in early infected and HPV-transformed cells. During the productive phase with infectious virions when the viral genomes are maintained as episomes in keratinocytes, HPVs need to avoid and/or suppress host immunity in order to establish a persistent infection. Thus, although the immunomodulating roles of HPV E6 and E7 in the setting of cell transformation, or quasi-infection[56] have been studied, there is a complete gap in the literature when it comes to the effects of HPV on the responses of KC when the episomal HPV genome is present.

To circumvent this problem we made use of an unique experimental set up in which primary keratinocytes expressing episomal HPV genes were used. When these cells are grown in an organotypic raft culture system, they show differentiation-dependent production of infectious viruses mimicking the situation like early natural infection of HPV[57]. In addition, we also infected primary keratinocytes with native HPV virions. Thus, in contrast to the published studies focused on the effect of HPV proteins on the

immune response during malignant transformation, our set up specifically allowed us to focus on the immune evasive effects of HPV infection at the early phase of infection.

The questions we wanted to answer were:

1) Do KCs express functional PRRs, how is this altered during KC differentiation, and is this altered upon infection of KCs with HPV?

2) Which (groups of) immune genes are deregulated in KCs upon HPV infection and if so what are the molecular mechanisms underlying the HPV effect?

We investigated this in uninfected and hrHPV-infected KCs using genome-wide analyses and biochemical approaches in Chapters 2 + 3.

3 Adaptive immunity against HPV infection

Adaptive immunity consists of T cells and B cells crucial for the formation of immunological memory enabling the immune system to respond rapidly and effectively to a specific pathogen that has been encountered previously. APCs capture HPV proteins and digest them into peptides. The APCs then migrate to the lymph nodes where HPV peptides are presented to the T cells. Simultaneously, DCs or macrophages expressing TLRs, NLRs, RLRs, C-type lectins [58] are activated by binding to the viral PAMPs through innate immune receptors present on cell surface. Moreover, CD4+ T cells recognizing their cognate peptides may activate DCs via CD40-CD40L interaction [59]. The activated immune cells release inflammatory cytokines including IL-1, IL-6, TNF-α, and IL-12, which induce local inflammation and function as chemoattractants to other immune cells or to polarize the immune response for instance to a TH1 (by IL-1, IL-12) type, important for the induction of adaptive immune responses.

After the recognition of an antigen, CD4 T cells may differentiate into for instance Th1, Th2 cells or T regulatory cells, largely determined by the cytokine milieu in the local microenvironment and the activation status of the DCs. T regulatory cells will suppress adaptive immune responses, Th1 cells promote cell-mediated immune responses, and Th2 cells sustain humoral effector responses. B cells are responsible for producing antibodies which function to neutralize and opsonize viral antigens. The growth, maturation, and production of antibodies by B cells are dependent on interaction with APCs and the cytokines profile secreted by CD4 helper T cells. HPV has many targets (E2, E6, E7, L1 and L2) against which antibodies can be generated during natural infection. While antibodies directed against E2, E6, E7 are weak and unable to mediate protective immunity against HPV infection, L1 and L2 capsid proteins are targets

of neutralizing antibodies and may prevent viral infection [60]. The majority of these antibodies are of the IgG1 class, a frequent response against viral antigens [61]. Eight to nine months after natural infection sero-conversion and neutralizing antibodies can be detected, but their levels are low, not apparent in all women [62], and not likely to prevent against subsequent infections.

The control of HPV infection requires an effective T cell response comprising both virus-specific CD8+ CTLs and CD4+ IL-2/IFN-γ-producing Th1 cells [63]. In healthy individuals, circulating HPV16-specific CD4+ Th1 and Th2 cells and CD8+ CTLs reactive to a broad array of epitopes in the viral early (E2, E6, E7) and late (L1) antigens can be detected that are able to migrate to areas where viral antigens are presented [63]. Furthermore, spontaneous regression of HPV-induced lesions is associated with the presence of circulating CD4+ and CD8+ T cells specific for HPV early antigens and coincident with the infiltration of the lesions by CD8+ CTLs and CD4+ T cells in numbers that surpass those of CD25+ Tregs [63].

By contrast, most individuals with HPV-induced progressive disease show an undetectable or a weak circulating T cell response to the HPV early antigens [64-68]. Additionally, progressive disease is associated with a loss of locally present IFN-γ and an increase in immunosuppressive IL-10 [69]. In addition, T cells expressing TGF-β have been detected in HPV-induced lesions [70]. IL-10 and TGF-β may directly suppress HPV-specific immunity since IL-10 can strongly inhibit the production of pro-inflammatory cytokines and TGF-β has a potent negative effect on the proliferation and Th1-differentiation of T cells [69]. Moreover, there is a steady increase in the number of tumor-infiltrating Foxp3+ Tregs , IDO+ cells, and macrophages [69], further suppressing the anti-tumor immunity. Intratumoral CD4+ and CD8+ T cells in this suppressive milieu generally lack the expression of granzyme B and thus are functionally impaired [71].

3.1 Mechanisms of escape of HPV from adaptive immunity

In hrHPV-induced cancer, HPV E7 downregulates the expression of the transporter associated with antigen protein (TAP1) which is essential in mounting MHC Class I presentation of HPV peptides by transformed cells [72, 73] resulting in suppression of HPV's antigen presentation s thereby impeding the recognition of transformed cells by effector CTLs. Additionally, HPV16 E5 downregulates MHC/HLA class I [74, 75].

The function of regulatory T cells (Tregs) is the induction of tolerance, but they also suppress anti-tumor responses. The number of Tregs is increased in HPV-induced tumors probably attracted by tumor-produced CXCL12 [76]. Tregs inhibit the

proliferation and cytokine (IFN-γ and IL-2) secretion of activated naïve CD4 T cells and Th1 cells [77]. As for APCs, Tregs alter their protein expression necessary for efficient antigen presentation and evoke the production of indoleamine 2,3-dioxygenase (IDO) by dendritic cells, which is an enzyme toxic to T cell populations [78]. Treg derived products like TGF-β, carbon monoxide, galectins, and IL-10 are considered to be immunosuppressive [79-83]. Notably, IL-10 producing HPV-specific Tregs highly capable of inhibiting the proliferation and cytokine (IFN-γ and IL-2) production of recently activated naïve CD4+ T cells, Th1 cells, and CD8+ CTLs have been isolated from premalignant lesions and cancer [66, 84] indicating that local immune suppression milieu may be a result of erroneously skewed HPV-specific T cell response. Importantly, high numbers of intratumoral Tregs are associated with poor prognosis of cervical cancer [85-87] implying role of Tregs in suppression of anti-tumor immunity.

3.2 T cell co-inhibitory molecules in cancer

Effective activation of T cells requires two signals: the first is mediated by the recognition of an antigen presented via the major histocompatibility complex (MHC) on antigen presenting cells (APC) by a corresponding antigen-specific T cell receptor (TCR). Second, co-signaling occurs via T cell co-signaling receptor molecules binding to ligand molecules expressed on the APCs, which can further enhance or dampen primary signaling pathways. Co-signaling is involved in all phases of T cell function including priming, activation, expansion, effector function and contraction.

The best characterized co-signaling molecules includes member of the CD28 and B7 superfamily, which are involved in both co-stimulatory and co-inhibitory processes. The interaction between CD28 and B7 family molecules are critical for immune response for infection and diseases [88-90]. For example, T cell activation depends on binding of CD28 to B7-1 (CD80) and B7-2 (CD86) on APCs while cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152), another member of CD28 family downregulates T cell activity by binding B7-1 and B7-2 [88-90].

Molecules of the B7-H1/PD-1 pathway are also critical modulators of the immune responses. Programmed Death-1 (PD-1, CD279) is a member of the CD28 family expressed on activated T cells, B cells, dendritic cells and macrophages [91]. PD-1 has two ligands B7-H1 (CD274, PD-L1) and B7-DC (CD273, PD-L2) of the B7 family. While B7-H1 expression is inducible on a variety of cell types in lymphoid and peripheral tissues, B7-DC is more restricted in myeloid cells including dendritic cells [91]. The major role of B7-H1/PD-1 pathway is to tune down inflammatory immune responses in order to protect tissues and organs from collateral damage.

Co-inhibitory molecules associated with inactivation of T cells include PD1, TIM3, CTLA4, CD160, LAG3 and 2B4 [92]. Initial studies indicated that PD-L1- PD1 was a crucial pathway in the regulation of CD8+ T cell exhaustion, as blockade of PD-L1- PD1 interactions in chronic infection or tumor microenvironment restored CD8+ effector function, whereas blockade of other individual co-inhibitory pathways alone (TIM3, CTLA4, and LAG3) showed less effective in rescuing T cells [93, 94]. However, combined blockade of PD-L1- PD1 with these co-inhibitors, notably has a synergistic effect in reversing T cell exhaustion [94-96]. More recently it was shown that the expression of these molecules are a sign of T-cell activation rather than exhaustion, only when the cognate ligands are expressed T-cell function may be downregulated [97]. Notably, the expression of multiple co-inhibitory receptors by T cells is associated with a progressive loss in proliferation, production of proinflammatory cytokines (IL-2, TNF-α and IFN-γ), cytotoxicity and the ability to become memory cells [94].

3.3 PD-1 is a co-inhibitory receptor that can be expressed on activated T cells

PD-1 is a 50-55 kDa type I transmembrane glycoprotein composed of an extracellular Ig domain and a 20 amino acid stalk. Its cytoplasmic tail contains two conserved tyrosine-based signaling motifs, an immunoreceptor tyrosine-based inhibition motif (ITIM), followed by an immunoreceptor tyrosine-based switch motif (ITSM), both of which are phosphorylated upon PD-1 engagement and can have inhibitory function [98]. PD-1 signaling interferes with the earliest tyrosine phosphorylation events in TCR signaling, thereby suppressed the activation of PI3K/Akt resulting in the inhibition of T cell expansion. Additionally, PD-1 engagement recruits both SHP-1 and SHP-2, SH-2-domain containing protein tyrosine phosphatases, which dephosphorylate and deactivate Ras-MEK-ERK and AKT pathways that ultimately result in cell cycle arrest of T cells [99].

The role of PD-1 in suppressing the antiviral response was first demonstrated by the rapid clearance of adenoviral infection in PD-1 knockout mice compared to wild type [100]. In contrast, Barber et al. [101] in the model of chronic LCMV infection showed that antigenic persistence resulted in high level of PD-1 expression on CD8 T cells which is associated with loss of effector function, and what they called an immune exhausted phenotype. A similar role for PD-1 has been reported in other chronic viral infection such as Hepatitis, SIV and HIV [102-104]. T cells chronically exposed to antigen within tumor microenvironment may also develop such a functionally inactive phenotype [92].

3.4 PD-1 in anti-tumor immunity

There is accumulating evidence that tumors exploit PD-1-dependent immune suppression for immune evasion. The aberrant expression of B7-H1 and B7-DC has been found in many solid tumors and hematological malignancies. Additionally, PD-1 expression on tumor infiltrating lymphocytes (TILs) has been reported, suggesting that these T cells might be functionally suppressed. Importantly, a strong correlation between B7-H1 expression on tumor cells and unfavorable prognosis has been demonstrated for many cancers including kidney, ovarian, esophageal, bladder, gastric and pancreatic cancers and melanoma [105]. In renal carcinoma, patients with high tumor and/or lymphocyte B7-H1 levels were 4.5 times more likely to die of their cancer than patients with low levels of B7-H1 expression [106]. Ovarian cancer patients with tumors positive for both B7-H1 and B7-DC showed dramatically lower survival rate than patients with tumors negative for both of these ligands (46% versus 83% for 5-year survival) [107]. New studies focus on the role of other co-inhibitory markers and how they are exploited by cancers to evade host immunity [89].

3.5 PD-1 and regulatory T cells (Tregs)

Tregs play a critical role in the maintenance of immune tolerance. CD4+ Foxp3+ Tregs are the most studied suppressive T cell population. The *Foxp3* knockout mice develop severe autoimmune conditions and the mutations of the human gene *FOXP3* is associated with fatal human immune dysregulation, polyendocrynopathy, enteropathy, X-linked syndrome (IPEX) [108, 109] indicating the crucial roles of Foxp3+ Tregs in peripheral tolerance. Foxp3+ Tregs can be divided into "natural" Tregs and "induced" Tregs. While "natural" Tregs develop as committed regulatory cells from the thymus, the "induced" Tregs arise in the periphery by polarization of naïve CD4+ T cells for instance when the microenvironment produces TGF-β and IL-2 [110] or IL-10 [111].

Tregs are often associated with solid tumors in both human and murine models. Increased number of Tregs is associated with a poorer prognosis in many human cancers [112]. Foxp3+ Tregs highly express PD-1 and B7-H1 [113]. Surprisingly, B7-H1 binding to PD-1 on natural Tregs has been shown to inhibit Treg suppressive function, whereas PD-1 ligation on the conventional T cells has been shown to promote their differentiation into induced Tregs [114-116]. The ability of PD-1 to deliver signal through B7-H1 on Tregs remain unclear. Moreover, the exact function of co-stimulatory and co-inhibitory molecules on both natural Tregs and induced Tregs needs further clarification [92].

3.6 Cancer immunotherapy targeting PD-1 and B7-H1

Monoclonal antibody-mediated immune checkpoint blockade of the inhibitory immune receptors CTLA-4, PD-1, and PD-L1 has shown to be successful in treating patients with advanced cancer [117]. Treatment with CTLA-4 blocking monoclonal antibody ipilimumab improved the overall survival of untreatable metastatic melanoma patients [118]. Strikingly, the durability of objective responses by CTLA-4 blockade leading to a possible cure for some patients has fuelled new enthusiasm on cancer immunotherapy in general.

Consequently, a number of clinical trials are examining the blockade of PD-1 by monoclonal antibody therapy (anti-PD-1 mAb; Bristol-Myers Squibb [New York, NY, USA], CureTech/Teva [yavne, Israel] and Merck [Boston, MA, USA]) and B7-H1 (anti-B7-H1 mAb; Bristol-Myers Squibb, Genentech [San Francisco, CA, USA]) in the treatment of refractory solid tumors. Two large Phase I trials blocking PD-1/B7-H1 interactions in patients with advanced cancers including melanoma and non-small cell lung cancer (NSCLC) have reported highly promising results. Topalian et. al. used an IgG4 monoclonal antibody (BMS-936558) that targets PD-1 while Brahmer et. al. used a monoclonal antibody that targeted B7-H1. Tumor response rates were between 18-28% for patients treated with anti-PD-1 antibody and 6-17% for the anti-B7-H1 antibody [119, 120]. Moreover, combined blockade of CTLA-4 (with ipilimumab) and PD1 (with nivolumab) in advanced melanoma was associated with rates of objective response that exceeded the previously reported results with either ipilimumab or nivolumab alone [121], albeit that in another study with the anti-PD-1 antibody lambrolizumab a similar response rate was observed [122].

3.7 Our questions

The transition from normal epithelium, via low grade and high grade lesions to cervical carcinoma is associated with locally present influx of CD4+ and CD8+ T cells [69], nevertheless, these T cells are not always able to mount an effective anti-tumor response. Several mechanisms could affect the efficacy of the T-cell response, in particular the presence of regulatory T cells, the presence of immune suppressive myeloid cells and/or the expression of co-inhibitory receptors and their ligands. Earlier our lab showed a role for intratumoral regulatory T cells in hampering T-cell reactivity [65, 84, 87, 123]and later the role of myeloid cells was addressed [85, 124]. Therefore, in Chapter 4 of this thesis, we focused our studies on the expression of B7-H1/PD-L1 and B7-DC/PD-L2 by the tumor cells and the expression of PD1 by CD4, CD8 and regulatory T cells.

4 Chemokines in cancer related inflammation

Chemokines are immune-cell attracting cytokines (about 8-17 kDa) that binds to and activate a family of chemokine receptor. So far, over 50 chemokines have been described. On the basis of the position of four conserved cysteine residues, the chemokines can be divided into four families (CXC, CX3C, CC and C). Functionally, chemokines can be divided into inducible or inflammatory chemokines and constitutively expressed or homeostatic chemokines. Inflammatory chemokines are critical for attracting a diverse set of effector leukocytes to inflammatory sites and as such they play a key role in the innate immune response by recruiting neutrophils, monocytes/macrophages, dendritic cells (DC), and natural killer (NK) cells. Homeostatic chemokines play major roles in migration of antigen-presenting cells (APC) and lymphocytes into the lymph node, as well as in migration of effector T cells to reach tissues, thus play critical roles for an effective adaptive immune response. Recently, chemokines and their receptors have been identified as mediators of chronic inflammation which play a key role in the tumor growth, angiogenesis, and metastasis.

4.1 Chemokines, tumor-associated leukocytes and tumor microenvironment

Cancer cells create a favorable microenvironment by interacting with the stromal cells (including cancer associated fibroblast, CAFs, mesenchymal stem cells, MSCs) and triggering the homing of leukocytes (including tumor-associated macrophases, TAMs, and tumor-associated neutrophils, TANs). Intratumoral CAFs secrete altered types of chemokines including CXCL12 which promotes cancer cell proliferation and angiogenesis by recruiting endothelial cells into carcinomas [125, 126]. Moreover, CAFs secrete TGFβ which in turn increase the levels of CXCR4 in cancer cells, thus activating CXCR4/CXCL12 axis resulting in increased proliferation of cancer cells by activating Akt [125]. Recent work has also shown that MSCs produce chemokines like CCL5, when they come in contact with cancer cells [127, 128]. Secreted CCL5 acts on CCR5 present at the surface of breast cancer cells promoting their metastasis to the lung [127]. Cancer cells can also recruit circulating cells including monocytes and macrophages to the tumor. CC chemokines, especially CCL2 and CCL5, are major attractants of monocytes and macrophages to the tumor microenvironment and their levels correlate with the number of the infiltrating myeloid cells [129-131]. Macrophages in tumors (TAMs) are usually of the M2-type, promote tumorgenesis and are associated with poor prognosis [124, 132, 133]. Lymphocytes particularly TH2 lymphocytes are the other major leukocytes found in cancers. Their recruitment is controlled by CC and CXC chemokines. Infiltrating TH2 lymphocytes are tumor promoting and are associated with poor prognosis [134]. In human cervical cancer, TAM secreted VEGF-C was proposed to be involved in peritumoral lymphoangiogenesis, and ultimately to lymphatic metastasis [135].

4.2 Tumor cell survival, and proliferation, tumor growth and progression.

The expression of chemokine receptors by tumor cells affects their own proliferation and survival [136, 137]. Early studies show that antibodies against CXCR2 inhibited melanoma cell growth in vitro implying the role of CXCR2 in tumor growth [138]. Similarly, CXCR4/CXCL12 chemokine/chemokine receptor pairs have been shown to be very efficient in enhancing tumor cell growth [139-141]. Additionally, the stable expression of CXCR7 was shown to increase the survival of breast cancer cells in vitro without affecting their in vitro proliferation [142]. Knockout of CXCR7 in cancer cells and use of the CXCR7 antagonist CCX754 reduces tumor growth in vivo. Altogether this suggests critical role for CXCR7 in growth and survival of cancer [142, 143]. Moreover, it has been reported that chemokines and growth factors can influence each other in some tumors. Estrogens increases the expression of CXC12 which activates CXCR4/CXCL12 signaling pathway that in turn promotes estrogen receptor transcriptional activity [144].

4.3 Tumor cell invasion and metastasis

The expression of chemokines and their receptors have been implicated in the distinct tropism of metastatic sites or cancer cells. The binding pairs CXCR4/CXCL12, CCL19-CCL21/CCR7 and CCL27/CCR10 are involved in metastasis in bone, lymph node and skin respectively [145] due to high concentrations of chemokines produced by the site of metastasis that attract cancer cells to these locations or through generating a gradient of chemokines by the tumor cells creating autologous chemotaxis and a continuous cycle of recruitment of the cancer cells actively promoting their own metastasis and tropism [146]. Pancreatic ductal adenocarcinoma cells have been shown to express high levels of CX3CR1 and migrate towards a gradient of its ligand, CXC3CL1, produced by neurons and nerve fibers causing the cancer cells to metastasize in brain [147]. Clinical studies showed that CCR9-expressing human melanomas metastasize to the small intestine which expresses high level of CCL25, the ligand for CCR9 [148]. T cell acute lymphocytic leukemia (T-ALL) shows high risk of metastasis in the central nervous system (CNS). Silencing of CCR7 or its ligand CCL19 is sufficient to inhibit CNS metastasis in T-ALL [149]. Additionally, cancer stem cells expressing CXCR4 have been identified at the invasive front of the tumor which determines that metastatic phenotype of individual tumors [150]. CXCR4 and CCR7 expression are associated with lymph node metastasis as well as poor prognosis in patients with cervical cancer [151-153]. The distribution and the intensity of expression of CXCL12, CXCR4, CXCL16, and CXCR6 increases as neoplastic lesions progress through CIN1, CIN2, and CIN3 to invasive cervical cancer. Moreover, among those molecules only CXCR6 is associated with long-term outcomes in that the patients with high CXCR6 expression had significantly shorter overall survival than those with

low CXCR6 expression and the expression of CXCR6 is associated significantly with lymph node metastasis [154].

4.4 Oncogene pathways involved in chemokine production and chemokine receptor signaling

Chemokines bind to G-protein-coupled receptors (GPCRs) and activate a series of downstream effectors and signal transduction pathways including PI3K, Jak-STAT [155]. In cancers, inactivating mutations of tumor-suppressors or activating mutations of oncogenes have been associated with deregulated production of chemokines in cancer cells which in turn cause tumor initiation and progression. Ras is frequently mutated in human cancers, which activates EGFR-ras-raf signaling pathways leading to the production of tumor-promoting chemokines including CXCL1 and CXCL8 [156, 157]. Myc is over-expressed in many human tumors and myc-activated tumor cells produce chemokines that can recruit mast cells to induce new vessel formation and tumor growth [158, 159]. Moreover, wild type p53 but not the cancer-specific mutants (R175H or R280K) represses CXCR4 expression resulting in reduced invasion of cancer cell lines through matrigel suggesting that mutation of tumor suppressor p53 increases the production of tumor-promoting CXCR4 [160]. Oncogenic changes not only produce tumor-promoting chemokines, but may also suppress homeostatic chemokines production. EGFR-Ras signaling in cutaneous tumor cells reduces their ability to express CCL27, resulting in impaired recruitment of anti-tumor T-lymphocytes to the site of tumors [161]. Transgenic mouse models, expressing the early genes from HPV 16 under the control of the human keratin 14 promoter, have shown that HPV-induced lesions release the chemokine CCL2 which enhances macrophage recruitment into tumors via CCR2 [162]. In human neoplastic cervical epithelial cells, HPV 16 E5, E6 and E7 oncogenes have been shown to induce the inflammatory cyclo-oxygenase (COX)-prostaglandin axis, by elevating the expression of COX-2 which is involved in oncogenesis [163]. These studies directly link HPV oncogenes with the activation of inflammatory cascades in promoting cervical cancer. Thus, HPV is not associated with inflammation at the initial phase of infection, however, it is likely that following HPV DNA integration and transformation, hrHPV-transformed cells drive dysregulated inflammatory cascades, such as the COX-prostaglandin pathway in transformed epithelial cells promoting immune cell infiltration, chronic inflammation and ultimately to tumor progression [164].

4.5 Our questions

Chemokine receptors have been studied in the context of outcome of cervical cancer patients [151] (and discussed above). Our genome-wide mRNA expression profiling data

showed that CXCR7 expression is upregulated in hrHPV-infected KCs. Hence, we wondered if CXCR7 was also expressed in HPV+ tumors and whether its expression would be associated with disease outcome. Therefore, we studied a series of cervical cancers for the expression of CXCR7 and the co-dependency of expression of its putative receptors EGFR and CXCR4, the expression of its predominant ligand CXCL12 as well as associations with clinical outcome in cervical cancer patients in Chapter 5.

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Chapter 2

Human Papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes

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Human Papillomavirus Deregulates the Response of a Cellular Network Comprising of Chemotactic and Proinflammatory Genes

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Abstract

Despite the presence of intracellular pathogen recognition receptors that allow infected cells to attract the immune system, undifferentiated keratinocytes (KCs) are the main targets for latent infection with high-risk human papilloma viruses (hrHPVs). HPV infections are transient but on average last for more than one year suggesting that HPV has developed means to evade host immunity. To understand how HPV persists, we studied the innate immune response of undifferentiated human KCs harboring episomal copies of HPV16 and 18 by genome-wide expression profiling. Our data showed that the expression of the different virus-sensing receptors was not affected by the presence of HPV. Poly(I:C) stimulation of the viral RNA receptors *TLR3*, *PKR*, *MDA5* and *RIG-I*, the latter of which indirectly senses viral DNA through non-self RNA polymerase III transcripts, showed dampening in downstream signalling of these receptors by HPVs. Many of the genes downregulated in HPV-positive KCs involved components of the antigen presenting pathway, the inflammasome, the production of antivirals, pro-inflammatory and chemotactic cytokines, and components downstream of activated pathogen receptors. Notably, gene and/or protein interaction analysis revealed the downregulation of a network of genes that was strongly interconnected by IL-1β, a crucial cytokine to activate adaptive immunity. In summary, our comprehensive expression profiling approach revealed that HPV16 and 18 coordinate a broad deregulation of the keratinocyte's inflammatory response, and contributes to the understanding of virus persistence.

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Introduction

Cervical cancer is the second most common cancer in women worldwide. More than 520,000 women are diagnosed with invasive cervical cancer each year [1]. Cervical and other anogenital carcinomas arise as result of an uncontrolled persistent infection with a high-risk type human papillomavirus (HPV), in particular types HPV16 and HPV18 [2,3]. A detectable cervicovaginal HPV infection in young women is close to 1–2 years [4] before it is cleared, suggesting that HPV can evade host immunity. Indeed, the infection cycle of HPV is one in which viral replication and release is not associated with overt inflammation [5,6] and HPV-specific adaptive immune responses are often weak or lacking in patients with progressive HPV infections [7–10].

Stratified squamous epithelia consist of undifferentiated (basal layer) and increasingly differentiated KCs. The basal KCs are the primary target of HPV infection [11]. In these cells, innate immunity acts as the first line of defense against invading viruses. KCs express pathogen recognition receptors (PRRs) including TLR9, which responds to viral DNA [12], as well as TLR3, protein kinase R (EIF2AK2), and the RNA helicases RIG-I (DDX58) and MDA5 (IFIH1), which recognize single-stranded and double-stranded RNA (dsRNA) [13]. Ligand binding to these PRRs leads to direct NF-kappa-B activation resulting in the upregulation of pro-inflammatory cytokines, and/or activation of type I interferon (IFN) response genes including transcription factors IRF3 and IRF7 regulating the production of antiviral cytokines [13–22].

Expression of specific viral oncoproteins, E6 and E7, is required for maintaining the malignant growth of cervical cancer cells [23]. To understand how HPV infection may alter KCs and evade PRR activation, direct protein interactions including the binding of the HPV E6 oncoprotein to IRF3 have been studied [24,25]. An OncoChip expression study showed that retrovirally expressed E6

and E7 efficiently downregulated type I IFN responses in keratinocytes, but surprisingly also upregulated the expression of pro-inflammatory cytokines [26]. Another early microarray study described downregulation of interferon-inducible genes in KCs containing episomal HPV type 31 [27]. These studies indicated that HPV-derived proteins could meddle with host immunity but the full spectrum of interference is within the limitations of these studies not visible.

We aimed at understanding the effects of high-risk HPVs on the immune response in KCs. First, we confirmed expression of the viral RNA receptors in undifferentiated and differentiated cells, while DNA sensor *TLR9* was restricted to differentiated cells, and showed that HPV does not interfere with expression levels of the PRRs. Next, we focused our studies on undifferentiated KCs, since these are the target cells for latent infection with HPV. We generated expression profiles of several different control KCs and KCs harboring episomal copies of entire HPV16 or 18 genomes [28,29] on microarrays representing 24,500 well-annotated transcripts to study differences in the baseline gene expression by the presence of HPV. In addition, we studied differences in response to triggering the viral RNA PRRs with the synthetic dsRNA poly(I:C). Although HPV is a DNA virus, non-self dsDNA can serve as template for transcription into dsRNA by polymerase III and induce type I interferon and NF-Kappa-B through the RIG-I pathway [30–32]. Here, we show that HPVs were able to dampen a network of genes associated with activation of the adaptive immune response encoding antimicrobial molecules, chemotactic and pro-inflammatory cytokines, and proteins that are involved in antigen presentation, and that most of them are interconnected via *IL1B*.

Materials and Methods

Ethics statement

The use of discarded human foreskin, cervical and vaginal keratinocyte tissues to develop cell lines for these studies was approved by the Institutional Review Board at the Pennsylvania State University College of Medicine and by the Institutional Review Board at Pinnacle Health Hospitals. The Medical Ethical Committee of the Leiden University Medical Center approved the human tissue sections (healthy foreskin, healthy cervix, HPV16- or 18-positive cervical neoplasias) used for staining. All sections and cell lines were derived from discarded tissues and de-identified, therefore no informed consent was necessary.

Cell culture

Human epidermal KCs were isolated from foreskin, vagina, or cervix of unrelated donors [33] and established on a layer of lethally ^{137}Cs-irradiated mouse 3T3 fibroblasts. Passage 4–5 of primary KCs - devoid of contaminating cells - were grown in serum-free medium (Defined KSFM, Invitrogen, Breda, The Netherlands). Partial differentiation was induced by 1.8 mM Ca2+ for 24 hrs, terminal differentiation by placing KCs in single-cell suspension into serum-free medium containing 1.75% methylcellulose and 1.8 mM Ca2+ for 24 hrs [33]. KC cell lines maintaining episomal copies of HPV16 and HPV18 were created via an electroporation technique described previously [28,29] but without antibiotic selection. The cell lines were 100% HPV-positive. Southern analyses confirmed the recircularization and subsequent maintenance of episomal viral genomes at approximately 50–100 copies per cell (data not shown). The HPV-positive lines growed at similar rates with population doubling times of ~2 days) and, when placed in raft culture, all underwent the late stages of the virus life cycle, such as genome amplification, late gene expression, and virus production (data not shown). HPV-positive cells were grown in monolayer culture using E medium in the presence of mitomycin C-treated 3T3 fibroblasts [28,29] for passage 6–7, and adapted to serum-free medium for one passage before experimentation. All cells used were tested and found free of mycoplasm. Where indicated, cells were stimulated with poly(I:C) (25 µg/ml, InvivoGen, San Diego, USA). CCL5 and IL-1B concentrations in supernatants were determined using the Quantikine ELISA kits (R&D Systems, Minneapolis, USA).

Immunohistochemistry

Standard immunohistochemical staining was performed using antibodies against human RNASE7 (Sigma-Aldrich, Zwijndrecht, Netherlands, dilution 1:1600) and TLR9 (clone 26C593.2, Imgenex, San Diego, USA, 1:800). Four-µm sections of formalin-fixed, paraffin-embedded tissues were deparaffinized, endogenous peroxidase was quenched with 0.3% H2O2 in methanol for 20 minutes, and antigen retrieval was performed by boiling the sections for 10 minutes in Tris-EDTA buffer (pH 9.0). For TLR9 antibody stainings, antigen retrieval was performed by boiling the sections for 10 minutes in citrate buffer (pH 6.0). Isotype control antibody against mouse IgG1 (1:1000 dilution, code X0931, DAKO, Glostrup, Denmark) was used. Primary antibodies were incubated overnight at room temperature. The Powervision detection system was applied (DAKO, Heverlee, Belgium). Mayer's haematoxylin was used for counterstaining of the slides.

Total RNA isolation and quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen, Breda, The Netherlands) followed by the RNeasy Mini Protocol (Qiagen, Venlo, The Netherlands). Total RNA (0.2 µg) was reverse transcribed using SuperScript III (Invitrogen) and oligo dT primers (Promega, Madison, USA). Triplicate PCR reactions were performed with 20 pmol of gene-specific primers and Taq DNA polymerase (Promega) using PCR conditions and primers as described previously for *TLRs* [34] and *SPRR2A* [35]. Predesigned primers and probe mixes for *TLR3*, *CCL5*, *IL1B*, *RNASE7*, *NLRP2*, and *GAPDH* were from Applied Biosystems (Foster City, USA). Threshold cycle numbers (Ct) were determined with 7900HT Fast Real-Time PCR System (Applied Biosystems) and the relative quantities of mRNA per sample were calculated using the ΔΔCt method with *GAPDH* as the calibrator gene. The relative levels of mRNA were determined by setting the mRNA expression level of the lowest expressing control KCs to 1, unless otherwise indicated.

cRNA synthesis and microarray hybridization

We used four primary KC cultures, HVKp1 and HVKp2 (both vaginal), HFKc1 and ESG2 (both foreskin), as well as four KC cell lines stably maintaining episomal HPV16 or 18, HVK16 (vaginal), HVK18 (vaginal), HCK18 (cervical), and HPV16 (foreskin). Cells were harvested at three conditions: unstimulated, 4 hrs and 24 hrs of 25 µg/ml poly(I:C). Total RNA for these 24 samples was isolated as stated above, and analyzed on an RNA 6000 Nano Lab-on-a-Chip in the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), showing RIN scores above 9.6. Total RNA (50–100 ng) was reverse-transcribed, amplified and biotin-labeled using the Ambion Illumina TotalPrep RNA Amplification kit (Applied Biosystems, Streetsville, ON, Canada). Concentration measurements were done using the NanoDrop ND-3300 (Isogen Life Science, De Meern, The Netherlands), 750 ng of labeled cRNA was hybridized to Sentrix HumanRef-8 V2 BeadChips (22K, Illumina, San Diego CA, USA), and scanned with BeadArrayer 500GX (Illumina). The samples were randomized

for two cRNA synthesis batches and (sub)array location. Raw probe level intensity values were summarized and exported with Illumina probe annotations using Illumina BeadStudio v3.2 (Gene Expression Module BSGX Version 3.2.7). Non-background corrected data were variance stabilizing transformed followed by robust spline normalization [36] using the lumi v1.6.2 [36,37] and lumiHumanAll.db v1.2.0 [38] BioConductor v2.2 packages in R v2.7.1 (R Development Core Team, www.R-project.org). All microarray data is MIAME compliant and the raw data has been deposited in the MIAME compliant database Gene Expression Omnibus with accession number GSE21260, as detailed on the MGED Society website http://www.mged.org/Workgroups/MIAME/miame.html.

Analysis of differential gene expression

We fitted a linear model in limma v2.14.7 [39] with 'virus' (HPV-positive) and 'stimulation' (4 and 24 hrs) effects. We used a nested variable within 'virus' for the individual cell lines, where HVKp1 and HVK16 were the reference cells for the HPV-negative and HPV-positive groups, respectively. Multiple-testing corrected p-values [40] and log2 fold changes were extracted for different contrasts. For Table S1, the 4 and 24 hrs timepoints were combined into one F-test in limma. One-dimensional hierarchical clustering of log2 fold changes derived from limma was done in Spotfire DecisionSite 9.1 v19.1.977 using correlation as similarity measure and complete linkage.

Functional genomics analyses

Functional annotation of the groups of co-regulated genes identified by hierarchical clustering was performed using Anni 2.0 [41]. We used GenMAPP v2.1 [42] to overlay expression on the TLR signaling pathway, which was based on automatic extraction from KEGG [43] hsa04620 (7/17/09) with improved layout using PathVisio v1.0 beta software [44]. The edited pathway is available from GenMAPP and WikiPathways [45].

We used CORE_TF (www.lgtc.nl/CORE_TF) based on TransFac 11.2 and Ensembl 49 [46] to identify over-represented transcription factor binding sites in promoters compared to a random set of 2966 promoters (1000 bp upstream+exon 1). Microarray probe EntrezGene IDs were converted to Ensembl Gene IDs using IDconverter [47], entries resulting in multiple or missing Ensembl Gene IDs were removed. The match cutoff was set to minimize the sum of false positives and false negatives; position weight matrices with a p-value for over-representation ≤0.01 and a frequency below 50% in the random set were selected.

The network was constructed using Ingenuity Pathways Analysis (IPA 7.6; Ingenuity® Systems, Inc., www.ingenuity.com). The 663 HPV signature genes were filtered for the more extreme log fold changes to obtain a gene signature strongly affected by HPVs, and to get the number of genes below 500, which is the maximum limit of IPA for making a network. Genes not connected were deleted, the remaining HPV signature genes that were initially excluded as stated above were included to generate the final network consisting of 212 connected genes. All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base.

Results

Expression of viral pathogen recognition receptors in KCs

We determined the mRNA expression of Toll-like receptors and retinoic acid-inducible gene I (RIG-I)-like receptors in undifferentiated, partially and fully differentiated KCs. Expression of the small proline-rich protein 2A (*SPRR2A*) was used as a molecular marker of KC differentiation (Fig. S1A). Undifferentiated KCs were found to express *TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR6*, *TLR10*, *RIG-I and MDA5* (Fig. 1A, 1B). Among the viral PRRs, *TLR7*, *TLR8* and *TLR9* were not detectable while *TLR3*, *RIG-I* and *MDA5* were expressed. In parallel experiments, transcripts of *TLR4* and *TLR7-9* were readily detected in mRNA samples from Ramos B-cells and monocytes (Fig. S1B). The expression in KCs is largely in line with previous reports by others [13]. HPV-positive KCs showed essentially the same pattern of PRR expression (Fig. 1A, 1B). Additionally, real-time RT-PCR showed similar levels of *TLR3* in HPV-negative and HPV-positive KCs (Fig. 1C). Upon differentiation KCs also expressed the DNA sensor *TLR9*, which was confirmed by immunohistochemistry in human foreskin and cervical epithelia (Fig. S2). TLR9 was also expressed in the differentiated layers of HPV-positive cervical epithelial neoplasias (Fig. S3). The absence of *TLR4* expression in differentiated KCs, which was confirmed by expression microarray (see below), is consistent with work by others showing that TLR4 was only found in HaCat cells, but not in primary human KCs [16,48]. The pattern of *TLR* expression in differentiated HPV-positive KCs was similar to that in HPV-negative cells. Thus, HPVs did not affect mRNA expression of the tested PRRs.

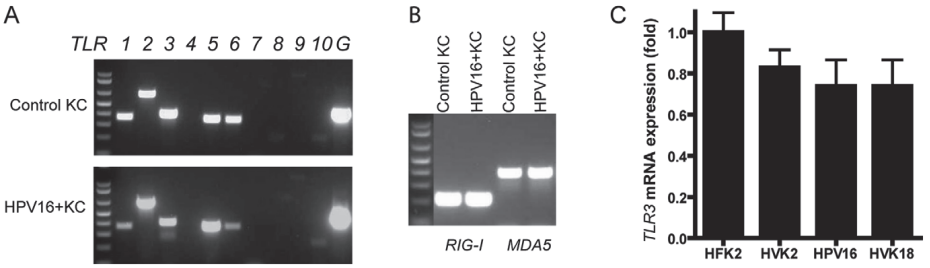


Figure 1. KCs express pathogen recognition receptors. Total RNA of indicated KCs was subjected to RT-PCR (35 cycles) with specific primers for human *TLR1-10, GAPDH* (indicated by a G) (A), *RIG-I* or *MDA5* (B). Control KC correspond to HFK2. Size markers (1 kb plus DNA Ladder, Invitrogen) from high to low: 1000, 850, 650, 500, 400, 300, 200, 100 bp; 1.8% agarose gel. (C), TaqMan RT-PCR results showing *TLR3* mRNA expression in HPV-negative (HFK2 and HVK2) and HPV-positive (HPV16 and HVK18) KCs. Fold-changes are relative to HFK2. Data are mean ± SD, n = 3.
doi:10.1371/journal.pone.0017848.g001

HPV signature genes

We subsequently studied whether HPVs affected the signalling of PRRs using genome-wide expression profiling. Control KCs (n = 4) and KCs with episomal HPV16 or HPV18 genomes (n = 4) of foreskin, vaginal or cervical origin from eight different individuals were used to include biological variation. Since HPVs infect basal KCs, we focused on the viral PRRs expressed in undifferentiated cells, including *TLR3*, *RIG-I* and *MDA5*, which respond to the synthetic dsRNA poly(I:C) [13]. In agreement with the RT-PCR data, the presence of HPV did not change the expression of these PRRs (Table S1).

To obtain a robust signature of genes affected by HPVs, we selected differentially expressed genes between HPV-positive and -negative KCs at 0, 4 or 24 hrs of poly(I:C) stimulation with a false discovery rate (FDR) of 0.05 (1529 probes). Furthermore, we applied an absolute log2-fold change filter ≥1 to select genes that were at least two-fold up- or downregulated (663 probes representing 634 unique genes), designated "HPV signature genes" (union of genes in Venn diagram Fig. 2A, Table S2). The majority of HPV-specific differentially expressed genes were shared between all three (213) or two (150) conditions, with most overlap between 0 and 4 hrs. Notably, 219 genes were changed in the virus-positive group only after 24 hrs of poly(I:C) stimulation, showing that the effect of HPVs was more pronounced after poly(I:C) stimulation.

Poly(I:C) response in control KCs

We first focused on the effect of poly(I:C) stimulation in control KCs. While after 4 hrs (Fig. 2B left) we found 123 differentially expressed probes that were mainly upregulated, the response was more balanced and involved over 700 genes after 24 hrs of stimulation (Fig. 2B right). Many genes were upregulated, including pathogen-sensing receptors (*RIG-I*, *MDA5*, *PKR*), adaptor molecules (*MYD88*, *TICAM1/TRIF*, *TICAM2/TRAM*), and interferon regulatory factors (*IRF1*, *IRF6*, *IRF7*), see Table S1. These results are similar to a previous report showing that poly(I:C) stimulation induces antiviral and inflammatory responses in KCs [13]. Overlay of differential expression after 24 hrs of poly(I:C) stimulation on the TLR signaling pathway (KEGG hsa04620) showed upregulation of the Jak-STAT signaling pathway, triggered by temporary upregulation of *IFNB1* after 4 hrs poly(I:C) through the *TRAF3/TBK1* signal transduction route, resulting in upregulation of STAT1 and chemotactic cytokines *CXCL10* and *CXCL11*. In addition, via *TRAF6* the NF-kappa-B signaling pathway was triggered, activating cytokines/chemokines *TNF*, *IL1B*, *IL6*, *IL8*, *CCL3*, *CCL4*, and *CCL5* (Fig. S4). The cytoplasmic RNA sensing receptors MDA5 and RIG-I, which are not shown in the TLR signaling pathway, initiate signaling pathways that differ in their initial steps from TLR3 signaling, but converge in the activation of TBK1 and NFKB [13,49].

Deregulation of poly(I:C) response in HPV-positive KCs

The differentially expressed genes in the HPV-positive cells upon poly(I:C) stimulation largely overlapped with those in control KCs (Fig. 2B). Next, we studied the effect of the virus in the context of the TLR signaling pathway. Activation of the TLR signaling pathway in HPV-positive KCs upon 24 hrs of poly(I:C) stimulation was largely similar to the response in control cells (Fig. S5). However, when directly comparing HPV-positive and –negative cells after 24 hrs of stimulation, relative downregulation of the adaptor *TICAM1* and several cytokines (*IL1B*, *IL6*, *CCL5/RANTES*) was evident. These results suggest that the dsRNA PRR signaling pathway is less activated in HPV-positive cells (Fig. S6).

Co-regulated genes downregulated by HPVs

We extended our analyses to the full set of HPV signature genes, and identified genes with similar expression patterns over the sample groups by unsupervised clustering (Fig. 2C, Table S2). The gene dendrogram was cut at six clusters to generate profiles of co-regulated genes (Fig. 2C, 2D). To identify transcription factors possibly involved in the coordinated expression changes, we analyzed the promoter sequences of the genes in each of these clusters for enrichment of predicted transcription factor binding sites [46].

The first three clusters contained genes that were downregulated in HPV-positive compared to HPV-negative cells. Binding sites for early growth response (EGR) family transcription factors, involved in differentiation and mitogenesis, were significantly enriched in these clusters (Table S3). Cluster 1 genes (164 probes), including inflammasome components (*NLRP2*, *PYCARD*), were downregulated in HPV-positive KCs irrespective of poly(I:C) stimulation. Many of these downregulated genes, including several others in expression clusters 2 and 3, are involved in epidermis development and KC differentiation, fitting with the biological effect of HPV in delaying differentiation [50]. Cluster 2 genes (194 probes), including antimicrobials (*DEFB103B*, *LOC728454*, *AQP9*, *RNASE7*, *SRGN*), antigen presenting molecules (HLA-A, *-B*, *-C*, *-G*, *HCP5*), pro-inflammatory cytokines and chemokines (*CCL5/RANTES*, *CSF2/GM-CSF*, *TGF-alpha*, *IL23A*), interferon-inducible genes (*IFI27*, *IFITM1*), and *TICAM1* showed lower expression in the group of unstimulated HPV-positive cells. Moreover, the upregulation of these genes at 24 hrs of poly(I:C) stimulation as found in control KCs was suppressed in HPV-positive cells. Plots with microarray log2 intensities for four probes, *CCL5/RANTES*, *IL1B* (cluster 3, see below), *TICAM1* and *RNASE7* show the HPV effect as well as the biological variation inherent to using KCs derived from different individuals and different tissues, combined with two different HPV types (Fig. 3A). Downregulation of *CCL5* and *TICAM1* was confirmed by qRT-PCR (Fig. 3B and 3D), and ELISA showed lower CCL5 secretion in HPV-positive KCs upon poly(I:C) stimulation (Fig. 3C). For the small number of cluster 3 genes (15 probes), including pro-inflammatory cytokines (*IL1B*, *IL1A*, *IL6*), baseline expression (most likely activated by serum components) and upregulation at 4 and 24 hrs of poly(I:C)-stimulation were suppressed in HPV-positive cells. These genes were already upregulated after 4 hrs of stimulation, and showed promoter enrichment of binding sites for Rel/NFKB family members and STAT5 (Table S3).

Interestingly, the majority of expression cluster 2 and 3 genes followed a similar pattern of suppressed poly(I:C) response, suggesting that many of these genes are downstream targets of PRR signaling. We focused on the antimicrobial molecule RNASE7, a member of the RNase A superfamily with broad-spectrum antimicrobial activity and ribonuclease activity [51,52], which was not known to be affected by viral infection. qRT-PCR confirmed *RNASE7* upregulation upon poly(I:C) stimulation in control KCs, and suppression of poly(I:C)-mediated upregulation in the presence of HPVs (Fig. 4A). Normal cervical epithelial cells expressed RNASE7 throughout the epithelia, and high expression was observed in the basal layer, the *in vivo* equivalent to undifferentiated KCs (Fig. 4B). In contrast, RNASE7 protein was not expressed in any of the layers of undifferentiated cells within a representative HPV-induced CIN3 lesion. These data suggest that by suppressing the gene activation of antimicrobial molecules such as RNASE7, HPVs evaded the innate antiviral responses of the host.

Co-regulated genes upregulated by HPVs

Clusters 4–6 contained genes that were specifically upregulated in the HPV-positive compared to HPV-negative cells. Cluster 4

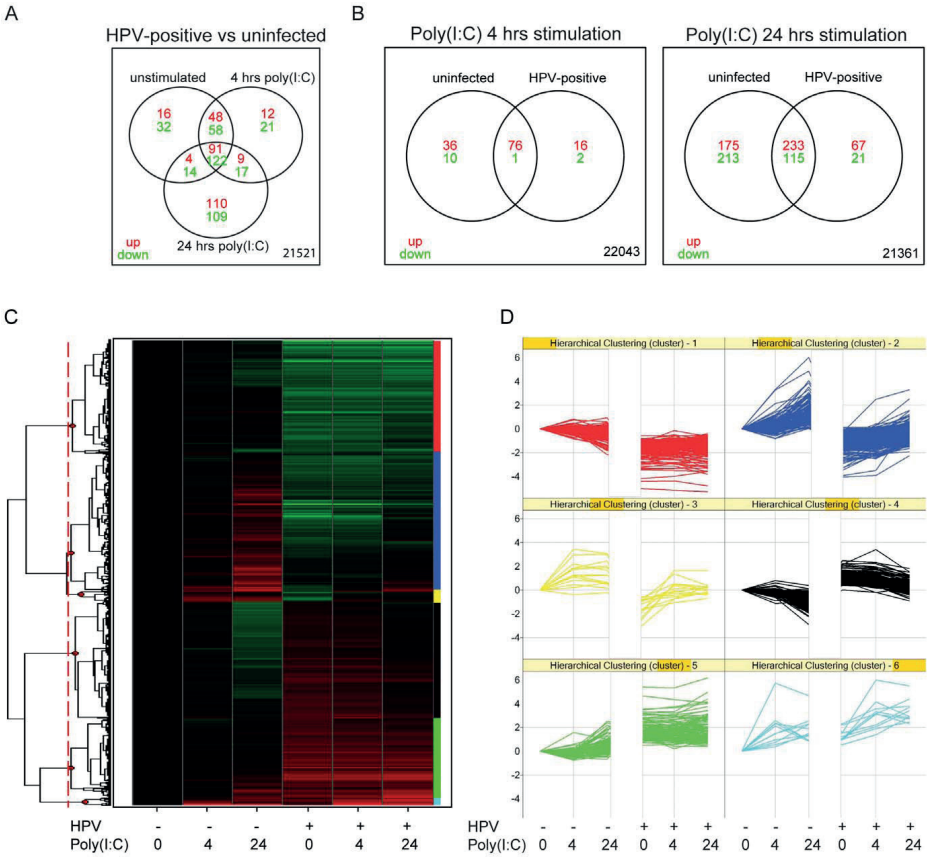
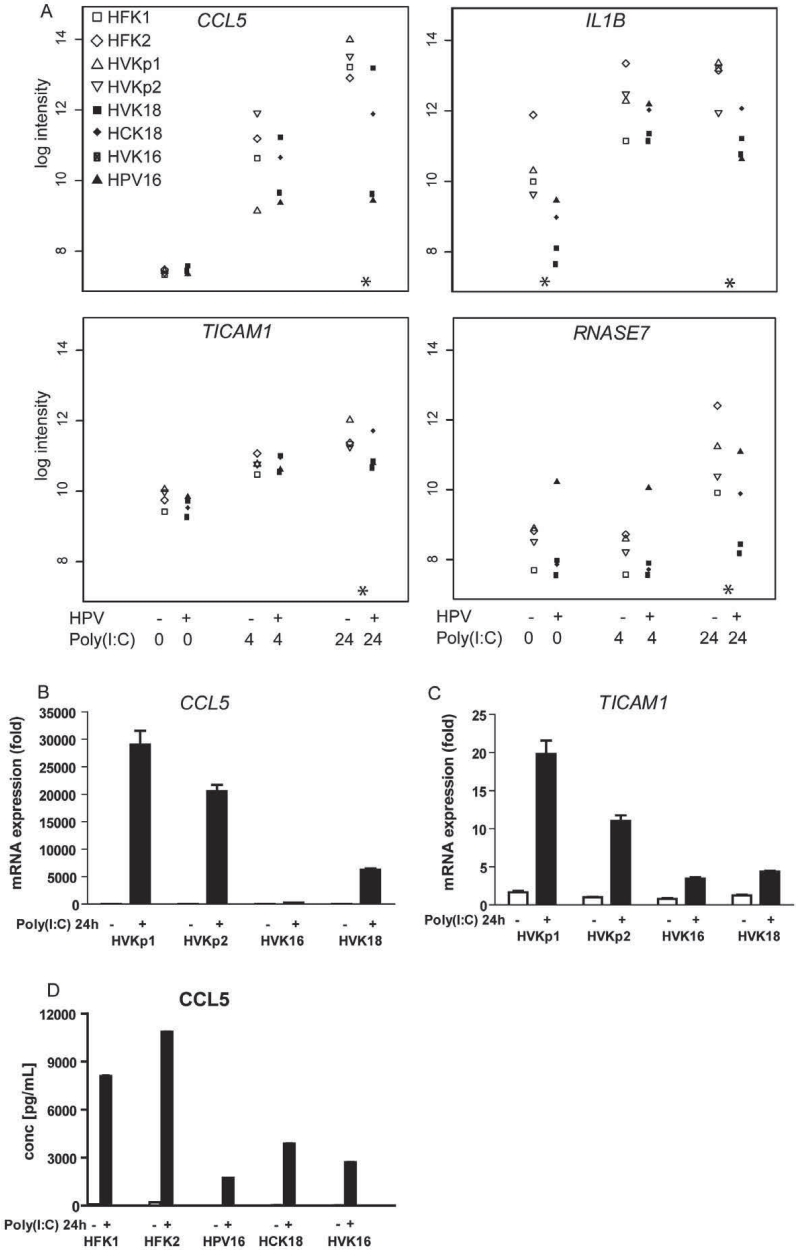


Figure 2. HPVs affect gene expression of KCs both at baseline and upon PRR stimulation. (A), Venn diagram depicting the overlap between 663 HPV signature genes with adjusted *p*-value≤0.05 and absolute log2-fold change≥1 altered by HPVs at baseline (unstimulated) and 4 and 24 hrs of poly(I:C) stimulation. Numbers in red represent upregulated genes while green indicates downregulated genes. (B), Venn diagrams showing the overlapping genes between control and HPV-positive KCs in their response to poly(I:C) stimulation for 4 hrs (left panel) and 24 hrs (right panel). Significance thresholds and colors as in (A). (C), One-dimensional hierarchical clustering of 663 HPV signature genes based on Pearson correlation using a complete linkage algorithm. Rows represent genes, columns represent ordered experimental groups each including four independent biological replicates. Limma log2-fold changes of the indicated conditions compared to the HPV-negative, unstimulated group are shown in the heatmap using red and green for up- and down-regulation, respectively. Black indicates no change. Six clusters based on cutting the gene dendrogram (red dashed vertical line) are indicated with color bars to the right. (D), Profile plots of co-regulated genes grouped according to the six expression clusters. Colors of the gene profiles match the bars to the right of the heatmap in (C). The y-axis shows the log2-fold change compared to HPV-negative, unstimulated KCs, the x-axis shows the ordered sample groups.
doi:10.1371/journal.pone.0017848.g002

genes (167 probes) included heat-shock response genes, cell cycle regulators and genes involved in replication initiation, transcription and splicing. These HPV-activated genes were downregulated upon poly(I:C) stimulation, but not to the same level as in control KCs. Binding sites for MEF2A, involved in the activation of stress-induced genes, and E2F, a family of transcription factors with a crucial role in the control of cell cycle that is indirectly activated by HPV E7, were enriched (Table S3). Cluster 5 (112 probes) contained cancer-related genes including tumor-promoting cytokines/chemokines and their receptors, e.g. *CXCR7*, of which the expression was higher in HPV-positive KCs irrespective of poly(I:C) stimulation. Many transcription factor binding sites were enriched, including motifs binding the oncoprotein MYC (Table S3). Finally, the smallest cluster 6 (11 probes) included several antiviral response genes (*TRIM5*, *ZC3HAV1*, *IFIT2*, *RARRES3*, *CXCL16*) that were stronger upregulated in HPV-positive than in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HFK1
HFK2
HVKp1
HVKp2
HVK18
HCK18
HVK16
HPV16
CCL5
IL1B
TICAM1
RNASE7
log intensity
HPV
Poly(I:C)
B
CCL5
mRNA expression (fold)
Poly(I:C) 24h
HVKp1
HVKp2
HVK16
HVK18
C
TICAM1
D
CCL5
conc [pg/mL]
HFK1
HFK2
HPV16
HCK18
HVK16

Figure 3. HPVs cause expression changes in immune-related genes. (A), Microarray log2 intensities (y-axis) for the expression levels of four example genes in HPV-negative and HPV-positive KCs, unstimulated or stimulated with poly(I:C) for 4 or 24 hrs. The eight individual KC cultures are color-coded. A star indicates a significant difference between HPV-positive and control KCs (see Materials and Methods for details). TaqMan RT-PCR showing *CCL5* (*RANTES*) (B) and *TICAM1* (C) mRNA expression in control (HVKp1 and HVKp2) and HPV-positive (HVK16 and HVK18) KCs at baseline and after poly(I:C) for 24 hrs. Data are mean ± SD, n = 3. (D), CCL5 secretion of control (HFK1 and HFK2) and HPV-positive (HPV16, HCK18, and HVK16) KCs measured by ELISA. Data are mean ± SD over three replicate samples.
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control KCs. Enriched binding sites included IFN-stimulated response element (ISRE), bound by transcription factor ISGF-3, and binding sites bound by interferon-response factors (IRFs).

In summary, the presence of episomal HPVs caused downregulation of genes involved in innate and adaptive immune responses as well as KC differentiation, while upregulated genes were involved in cell cycle, RNA and DNA metabolism. Overall, these data showed that HPVs induced coordinated changes in KC gene expression, detectable in unstimulated 'baseline' cells (mainly expression clusters 1, 5, majority of cluster 4) or after poly(I:C) stimulation (mainly expression clusters 2, 3, 6).

HPVs deregulate cellular networks

Understanding the network topology of gene and/or protein interactions may identify highly interconnected gene "hubs" targeted by HPVs. Therefore, we explored connections among the HPV signature genes based on literature and high-throughput database information collected in Ingenuity Pathways Analysis [53]. On the resulting network of 212 genes, we overlaid the expression log2-fold changes of HPV-positive versus control KCs after 24 hrs of poly(I:C) stimulation (Fig. 5). The center of the network was formed by the most interconnected gene *IL1B*, necessary for activation of the adaptive immune response [54], and *IL6*. *IL1B* and *IL6* were downregulated, and connected to genes encoding cytokines and antigen presentation molecules that were also lower expressed in HPV-positive cells. We studied *IL1B* in more detail, since it represented a central target for HPV-mediated suppression of both the innate and adaptive immune responses of KCs. RT-PCR data validated the microarray data showing that both the baseline and PRR-stimulated levels of *IL1B* were downregulated in HPV-positive KCs compared to control cells (Fig. 6A). Also, both the baseline and PRR-stimulated IL-1β secretion was lower in HPV-positive KCs (Fig. 6B). Secretion of IL-1β requires activity of both the TLR/NF-kappa-B and the inflammasome pathways [55]. The TLR/NF-kappa-B pathway activates pro-IL-1β expression, which is cleaved to active IL-1β by the inflammasome. In addition to the downregulation of pro-IL-1β, HPVs specifically downregulated the genes encoding inflammasome components *NLRP2* in three of the four HPV-positive lines (Fig. 6C) and *PYCARD/ASC*, but not *NALP3*, possibly contributing to the observed lower level of IL-1β. The most interconnected upregulated gene of the network was *CDKN2A*, involved in cell cycle progression. Thus, by targeting highly interconnected genes, HPVs reprogrammed the gene network of KCs in favor of immune escape and cell proliferation of HPV-positive cells.

Discussion

We studied systematic differences in genome-wide expression profiles of control and HPV-positive undifferentiated (basal) KCs focusing on immune-related effects. The parallel analysis of several control and HPV16- and 18-positive KCs from several genital tissues ensured that the results can be generalized. The HPV-positive KCs expressed the full array of HPV genes and mimic latent HPV infection *in vivo*, which is also reflected by the fact that these cells display the entire differentiation-dependent HPV life cycle upon culture in organotypic raft cultures [28,29]. Our studies revealed that while KCs are well equipped to respond to viral pathogens, latent infection with HPV results in suppression downstream of the PRRs as reflected by lower expression levels of effector molecules involved in innate and adaptive immune response.

No difference was observed in expression levels of viral RNA PRRs *TLR3*, *TLR9*, *RIG-I*, *MDA5* and *PKR* between control and HPV-positive KCs. We found that viral DNA PRR TLR9 was lacking in the basal layers in stratified squamous epithelia, but expressed in the suprabasal layers of the non-neoplastic epithelium. Previous studies suggested that E6/E7 expression affected

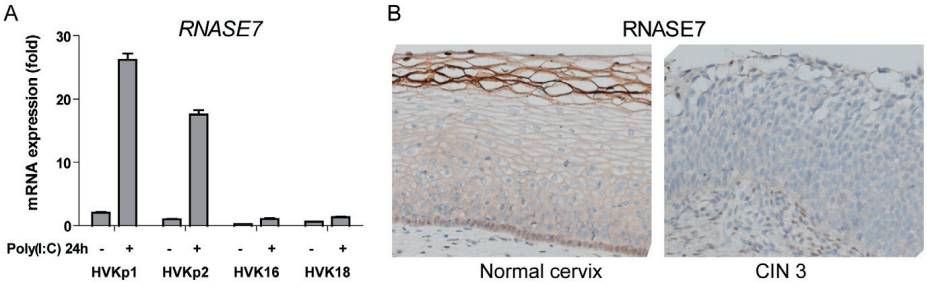

Figure 4. HPV inhibits RNASE7 expression in stimulated KCs and cervical neoplasia. (A), TaqMan RT-PCR showing *RNASE7* mRNA expression in control (HVKp1 and HVKp2) and HPV-positive (HVK16 and HVK18) KCs. Data are mean ± SD, n = 3. (B), RNASE7 protein is downregulated in cervical intraepithelial neoplasia 3 (CIN3). Immunohistochemical staining of paraffin-embedded sections showing RNASE7 protein expression in normal healthy ectocervical epithelium (left) and CIN3 (right). Original magnification 125×. Stainings shown are representative of at least three samples of different individuals.
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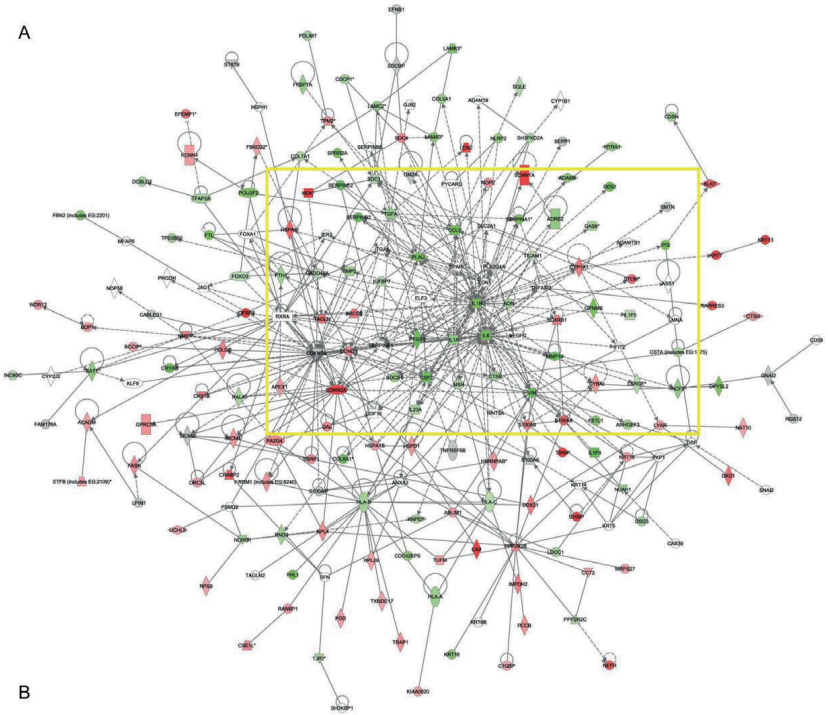
A



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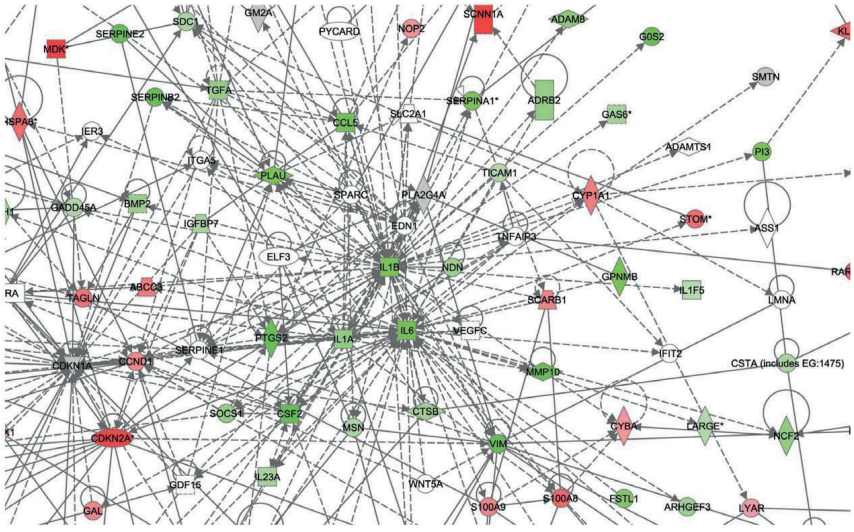
SDC1
GM2A
PYCARD
NOP2
SCNN1A
ADAM8
G0S2
SERPINE2
MDK*
TGFA
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SERPINA1*
ADRB2
GAS6*
SMTN
HSPA8
ER3
CCL5
SLC2A1
ADAMTS1
PI3
ITGA3
PLAU
TICAM1
CYP1A1
SPARC
PLA2G4A
GADD45A
BMP2
IGFBP7
EDN1
TNFAIP3
STOM*
ASS1
ELF3
IL1B
NDN
GPNMB
IL1F5
TAGLN
ABCC3
SCARB1
LMNA
IL6
VEGFC
PTGS2
IL1A
IFIT2
CSTA (includes EG:1475)
CDKN1A
CCND1
SERPINE1
MMP10
SOCS3
CSF2
CTSB
MSN
VIM
CYBA*
LARGE*
NCF2
CDKN2A
GDF15
IL23A
WNT5A
S100A9
S100A8
FSTL1
ARHGEF3
LYAR
GAL

Figure 5. HPVs deregulate a gene network in KCs. A network was constructed of 212 connected HPV signature genes using interaction data curated from literature and high-throughput screens in Ingenuity Pathways Analysis. (A), Overlay with gene expression changes of 24 hrs of poly(I:C)-stimulated HPV-positive KCs versus 24 hrs of poly(I:C)-stimulated HPV-negative KCs. (B), Zoom-in to central region of the network highlighting highly interconnected genes. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). Green, downregulated genes; red, upregulated genes; gray, not differentially expressed at the 24-hrs comparison; solid line, direct interaction; dashed line, indirect interaction.
doi:10.1371/journal.pone.0017848.g005

neither the expression nor the function of TLR9 [17], whereas others reported that E6/E7 expression resulted in loss of TLR9 expression [12]. Our data showed that forced differentiation of HPV-positive KCs resulted in the expression of TLR9, however, as HPVs inhibits differentiation this may appear as TLR9 loss similar to what was seen previously [12]. Thus, TLR9 is absent in the cells targeted by HPV, but other viral PRRs are expressed, including RIG-I that has been shown to indirectly function as a PRR for DNA viruses [30–32], suggesting that in essence undifferentiated KCs can sense HPV infection.

As there were no overt differences in the expression levels of PRRs, we focused on the interference of HPVs with the downstream pathogen-sensing machinery. First, our data showed that HPVs downregulated genes that have a direct antimicrobial function. Moreover, the presence of HPVs was associated with the downregulation of an array of pro-inflammatory and chemotactic cytokines, and antigen-processing and presenting molecules, and IL-1β and IL6 were the hubs in the center of this HPV signature gene network. Notably, the expression level of most of these genes was already lower at baseline. Poly(I:C), which triggers viral PRRs including TLR3 and importantly also RIG-I, increased their expression level in HPV-positive KCs albeit not to the same level as in control KCs. Previously it was shown that HPV31-positive KCs responded less well to interferon stimulation [27] and this fits with our own data showing that interferon-inducible genes (cluster 2) are downregulated. Apparently, this is not the only immune signaling pathway that is downregulated by HPV as our data reveal that also the TLR and the RIG-I-like receptor signaling pathways are suppressed in HPV-positive KCs. Notably, the failure of HPV31-positive KCs to respond to interferon was associated with downregulation of STAT1 (25). Specific down-regulation of STAT1 was found only in our HPV16-positive KCs

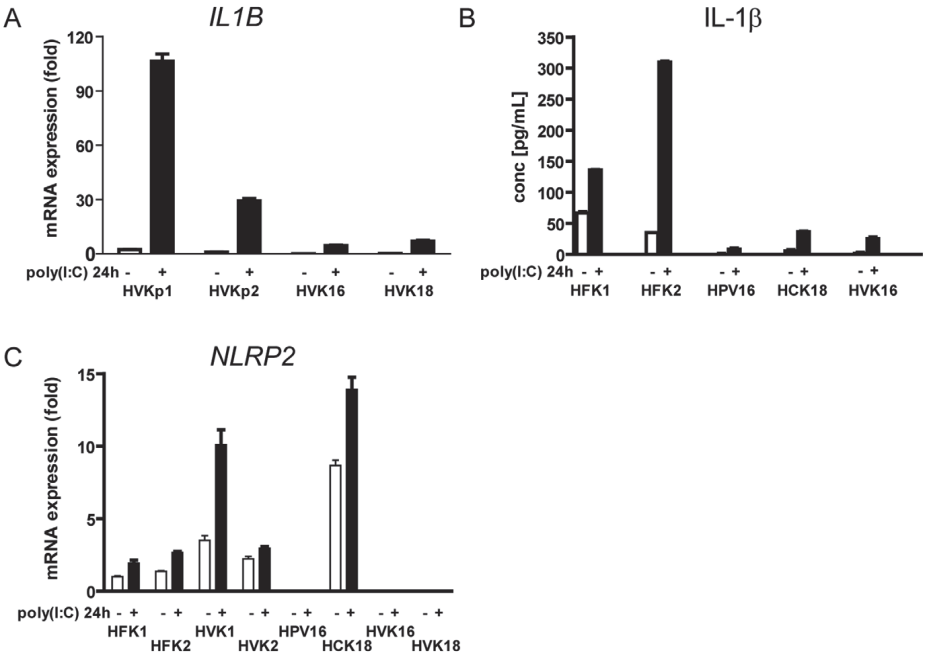

Figure 6. HPVs downregulate IL1B and inflammasome components. (A), TaqMan RT-PCR showing pro-*IL1B* mRNA expression in control (HVKp1 and HVKp2) and HPV-positive (HVK16 and HVK18) KCs. (B), IL-1β protein secretion of control (HFK1 and HFK2) and HPV-positive (HPV16, HCK18 and HVK16) KCs as measured by ELISA. (C), TaqMan RT-PCR showing *NLRP2* mRNA expression in HPV-negative (HFK1, HVK1, HVK2, HFK2) and HPV-positive (HPV16, HCK18, HVK16 and HVK18) KCs. In all three panels, data are mean ± SD, n = 3.
doi:10.1371/journal.pone.0017848.g006

(data not shown) suggesting that there may be a number of type-specific interactions with the host's immune system. Together these data suggest that HPVs dampen but do not block PRR signaling, and imply that the attraction of innate immune cells to the site of HPV infection, the subsequent initiation of adaptive immunity as well as the recognition of HPV-infected KCs is slowed down but not prevented. This clearly corresponds with the fact that it may take months or even a year to control HPV infections [4], and the increase in HPV-infected subjects capable of mounting an HPV-specific immune response in time [56]. Furthermore, it fits with the detection of HPV-specific memory responses after infection [9,57,58].

In particular, we found that HPVs downregulated toll-like receptor adaptor molecule 1 (*TICAM1*), a critical molecule in the TLR3 pathway that mediates NF-kappa-B and interferon-regulatory factor (IRF) activation via downstream molecules TRAF3, TRAF6 and RIP1 [59]. Notably, the other poly(I:C) recognizing PRRs also malfunction in HPV-positive KCs suggesting that HPVs affect the TBK1 and NF-kappa-B signaling pathways downstream of the PRRs and implying that downregulation of *TICAM1* is just part of the immune evasion strategy of HPVs. This is also illustrated by our finding that HPVs downregulated inflammasome components – needed to convert pro-IL-1β to the active form of IL-1β [60] - contributing to the lower secretion of IL-1β by HPV-positive cells. Of all candidate downstream targets IRF1 [25], IRF3 [24], the coactivator CPB [61], the IkB kinase complex [62], and the interferon-stimulated gene factor 3 (ISGF3) transcription complex [63] have been named as targets for either E6 and E7 proteins of HPV responsible for downregulating NF-kappa-B and TBK1 signaling. Others, however, have shown that E6 – instead of downregulating - may promote NF-kappa-B signaling [26,64]. Importantly, all of these studies relied on the overexpression of either one or both oncoproteins, which is more relevant for our understanding of HPV-transformed cells. The strength of our study lies in the use of KCs with episomal expression of the full array of HPV genes reflecting latent infection [28,29]. It would be of great importance to perform a genome-wide study of HPV-positive KCs during differentiation and interaction with (innate) immune cells thereby closely mimicking the situation *in situ*, but such an experiment would be technically challenging.

Non-cleared infection with high-risk HPVs leads to cervical and other anogenital carcinomas in which the virus genome integrates in the host genome [2,3]. The replication cycle of the virus is tightly coupled to the differentiation of basal KCs to stratified squamous epithelia and it is well known that HPVs inhibit KC differentiation [11]. In our expression data, this was reflected by concerted upregulation of cell cycle regulators and DNA/RNA synthesis, and downregulation of epidermis development and KC differentiation genes. *CDKN2A*, a critical cell cycle regulator upregulated by HPVs, was identified as one of the highly-connected hub genes in the network of HPV signature genes. Similar results were described by Nees et al. using a cDNA oncochip [26].

We have shown that HPV16 and 18 dampen a cellular immune-related network in HPV-positive KCs, and affect a much broader spectrum of PRR responses than the previously described IRF route. Our study provides a framework for future exploration into the molecular mechanisms involved in HPV-downregulated immunity. The biological variation in gene expression between different donors might reflect genomic variation that could play a role the balance between clearance and persistence of HPV. Additionally, it would be of interest to study if other viruses capable of causing persistent infection or low-risk HPVs that cause benign genital warts use similar mechanisms to escape host's immune responses.

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Author Contributions

Conceived and designed the experiments: SHvdB CJMM G-JBvO RO RK JMB. Performed the experiments: RK CM CB KL. Analyzed the data: RK SHvdB JMB. Wrote the paper: RK SHvdB JMB. Critical revision of the manuscript: RO CJMM GJBvO CM CB KL.

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Chapter 3

Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response

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Human Papillomavirus (HPV) Upregulates the Cellular Deubiquitinase UCHL1 to Suppress the Keratinocyte's Innate Immune Response

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Abstract

Persistent infection of basal keratinocytes with high-risk human papillomavirus (hrHPV) may cause cancer. Keratinocytes are equipped with different pattern recognition receptors (PRRs) but hrHPV has developed ways to dampen their signals resulting in minimal inflammation and evasion of host immunity for sustained periods of time. To understand the mechanisms underlying hrHPV's capacity to evade immunity, we studied PRR signaling in non, newly, and persistently hrHPV-infected keratinocytes. We found that active infection with hrHPV hampered the relay of signals downstream of the PRRs to the nucleus, thereby affecting the production of type-I interferon and pro-inflammatory cytokines and chemokines. This suppression was shown to depend on hrHPV-induced expression of the cellular protein ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) in keratinocytes. UCHL1 accomplished this by inhibiting tumor necrosis factor receptor-associated factor 3 (TRAF3) K63 poly-ubiquitination which lead to lower levels of TRAF3 bound to TANK-binding kinase 1 and a reduced phosphorylation of interferon regulatory factor 3. Furthermore, UCHL1 mediated the degradation of the NF-kappa-B essential modulator with as result the suppression of p65 phosphorylation and canonical NF-κB signaling. We conclude that hrHPV exploits the cellular protein UCHL1 to evade host innate immunity by suppressing PRR-induced keratinocyte-mediated production of interferons, cytokines and chemokines, which normally results in the attraction and activation of an adaptive immune response. This identifies UCHL1 as a negative regulator of PRR-induced immune responses and consequently its virus-increased expression as a strategy for hrHPV to persist.

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Introduction

Human papillomaviruses (HPVs) are absolutely species-specific small double-stranded DNA viruses. Persistent infections with a number of HPVs, predominantly types 16 and 18, can induce cancers of the anogenitalia as well as of the head and neck region. These so-called high-risk HPVs (hrHPVs) are widespread within all human populations where they are commonly transmitted by sexual contact [1]. The undifferentiated keratinocytes of the squamous epithelia are the primary target for hrHPV [2] where it establishes an infection that can last for up to 2 years, indicating that hrHPV has evolved mechanisms to effectively evade the innate and adaptive immune mechanisms protecting the majority of immunocompetent hosts [3,4].

Viruses and microbes contain pathogen-associated molecular patterns that are recognized by the host's pattern recognition receptors (PRRs), comprising the Toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) [5]. While all of these receptors activate signaling cascades that lead to activation of NF-κB via the canonical route, only RLRs and some TLRs activate interferon regulatory factors (IRFs) which induce the production of type I interferons (IFN) and other effector molecules [6]. The signals from the PRR to the cell nucleus are coordinated via ubiquitination, including that of the different tumor-necrosis factor receptor-associated factors (TRAFs) and the NF-κB essential modulator (NEMO). Poly-ubiquitination of TRAF and NEMO allows downstream signaling whereas disassembly of the formed poly-ubiquitin chains by deubiquitinating enzymes provides a mechanism for downregulating immune responses [6,7].

Author Summary

A persistent infection with high-risk human papillomavirus (hrHPV) may cause cancer. Whereas keratinocytes – the cells infected by hrHPV – are equipped with different receptors allowing them to recognize invading pathogens and to activate the immune system, hrHPV has developed ways to evade the host's immune response for sustained periods of time. We showed that hrHPV accomplishes this by interfering with the signaling of the pathogen receptors, thereby hampering the production of cytokines that are known to attract and activate the immune system. HrHPV accomplishes this by upregulating the expression of a cellular protein called ubiquitin carboxyl-terminal hydrolase L1 (UCHL1). This protein suppresses the activation of signals downstream of the pathogen receptor leading to reduced transcription factor activation and downstream gene expression, in particular that of type I interferon and pro-inflammatory cytokines. This lowers the attraction of immune cells and thereby the chance of hrHPV-infected cells to be recognized and eliminated and as such enables hrHPV to persist.

Keratinocytes (KCs) express TLRs 1–3, TLR5, TLR6, TLR10, RIG-I, protein kinase R (PKR), and MDA5 independent of their differentiation status and gain the expression of TLR9 upon full differentiation indicating that these cells may respond to pathogenic challenges [8,9,10]. Thus, KCs should be able to sense the presence of hrHPV genomic DNA directly via TLR9 or indirectly via RIG-I [5,11,12]. The expression levels of these PRR were not altered in hrHPV+ KCs [10]. However, via genome-wide expression profiling of keratinocytes activated through TLR3, PKR, RIG-I and MDA-5 we found that the presence of hrHPV dampens a network of genes encoding chemotactic, pro-inflammatory and antimicrobial cytokines suggesting that HPV's immune evasion strategy may rely on countering PRR-mediated cell signaling [10].

To understand the mechanisms underlying hrHPV's capacity to dampen PRR signaling we utilized a system that resembles the natural infection with HPV as closely as possible. It comprises the use of primary KCs that stably maintain the hrHPV genome as episomes following transfection. These hrHPV+ KCs grow at similar rates as non-transfected KC and have been shown to mimic HPV infection *in vivo* as they undergo the entire differentiation-dependent HPV life cycle documented by genome amplification, late gene expression, and virus production, upon culture of hrHPV+ KCs in organotypic raft cultures [13,14,15]. In addition, we used non-infected primary KC cultures and primary KCs newly infected with authentic HPV16 virions. These primary KCs were compared with respect to PRR signaling under different conditions and resulted in the identification of the cellular enzyme ubiquitin carboxyl-terminal hydrolase L1 (*UCHL1*) that was specifically upregulated by hrHPV in primary keratinocytes to dampen innate immunity. UCHL1 acted on the PRR-signaling pathway adaptor molecules TRAF3 and NEMO and its inhibition restored PRR-induced production of IFNβ and pro-inflammatory and chemotactic cytokines.

Results

High risk HPV is associated with a decreased induction of type I IFN and pro-inflammatory cytokines following stimulation of keratinocytes via different pattern-recognition receptors

Undifferentiated uninfected primary KCs and hrHPV+ KCs were tested for their capacity to respond to triggers of innate immunity by incubation with Pam3CSK4 (TLR1/2), poly(I:C) (TLR3, RIG-I, PKR and MDA-5) [9], lipopolysaccharide (LPS, TLR4), flagellin (TLR5), R848 (TLR7/8), or CpG (TLR9). The supernatant of non-infected keratinocytes contained higher levels of MIP3α and IL-8 but not MIP1α than hrHPV+ KCs at the basal level. Activation with poly(I:C) induced the production of high amounts of MIP3α, IL-8 and MIP1α in KCs but not in hrHPV+ KCs. Flagellin especially triggered the production of MIP3α by KCs but not in hrHPV+ KCs, although IL-8 was still produced (Figure 1A). The function of TLR9, expressed only at high protein levels in differentiated keratinocytes as measured by immunohistochemistry [10] and by RT-qPCR (Figure 1B), was tested by the capacity of CpG oligodeoxynucleotides (CpG ODN) to trigger the expression of mRNAs of pro-inflammatory cytokines and chemokines. Because suspension in methyl cellulose – to differentiate keratinocytes – does not allow the harvest of supernatant, secreted protein levels could not be measured. However, the experiments clearly showed that CpG ODN-stimulation resulted in the gene expression of *IFNB1* (*IFNβ*), *IL-8* and *CCL20* (*MIP3α*) in differentiated KCs but not in undifferentiated KC cultures (Figure 1C). As a control, KCs were also stimulated with poly(I:C) as TLR3, RIG-I and MDA-5 expression is independent of KC differentiation [10] and this resulted in the induction of pro-inflammatory cytokine expression in both undifferentiated and differentiated KCs (Figure S1). In contrast to differentiated uninfected KCs, the hrHPV+ KCs that expressed TLR9 after differentiation, failed to induce the expression of *IFNβ, IL-8 and MIP3α* upon incubation with CpG (Figure 1C), indicating that PRR-signaling can be suppressed in undifferentiated and differentiated hrHPV+ KCs.

As the basal KCs are the target for hrHPV and TLR9 is not functionally expressed in basal KCs and hrHPV+ KCs displayed an impaired production of cytokines in response to poly(I:C), subsequent studies were performed in the context of poly(I:C) stimulation. In addition to the secretion of cytokines, also the gene expression levels of *MIP3α*, *CCL5* (*RANTES*) and *IFNβ* in hrHPV+ KCs were lower when compared to uninfected KCs upon 3 or 24 hours of poly(I:C) stimulation (Figure 2A).

The production of pro-inflammatory cytokines and chemokines upon activation of the NF-κB pathway requires the phosphorylation and nuclear translocation of the subunit p65 [6]. The levels of phosphorylated p65 were lower in poly(I:C) stimulated hrHPV+ KCs than in non-infected KCs (Figure 2B), suggesting that the functional impairment of PRR signaling occurs upstream of this molecule. The IKK complex is a key component of the poly(I:C)-induced NF-κB pathway, with NEMO (IKKγ) functioning as a scaffold. The degradation of NEMO may form a mechanism for viruses to avoid innate immune signaling [16,17]. Therefore, the effect of hrHPV on the protein levels of NEMO was analyzed. Following treatment of non-infected KCs and hrHPV+ KCs with cycloheximide (CHX) – to prevent new protein synthesis – it became clear that NEMO degradation was enhanced in hrHPV+ KCs (Figure 2C and Figure S2), thereby explaining the decreased phosphorylation of p65 observed.

The production of type I IFN (*e.g.* IFNβ) requires the activation of cytosolic IRF3 by phosphorylation and subsequent translocation to the nucleus. Analysis of poly(I:C) stimulated KCs and hrHPV+ KCs suggested that also the levels of phosphorylated IFR3 levels were decreased in HPV+ KCs (Figure 2D).

The high risk HPV viral transcript is needed to impair PRR signaling

To confirm that the impairment in the production of IFNβ and pro-inflammatory cytokines did not simply reflect biological differences between the different primary KCs used but indeed

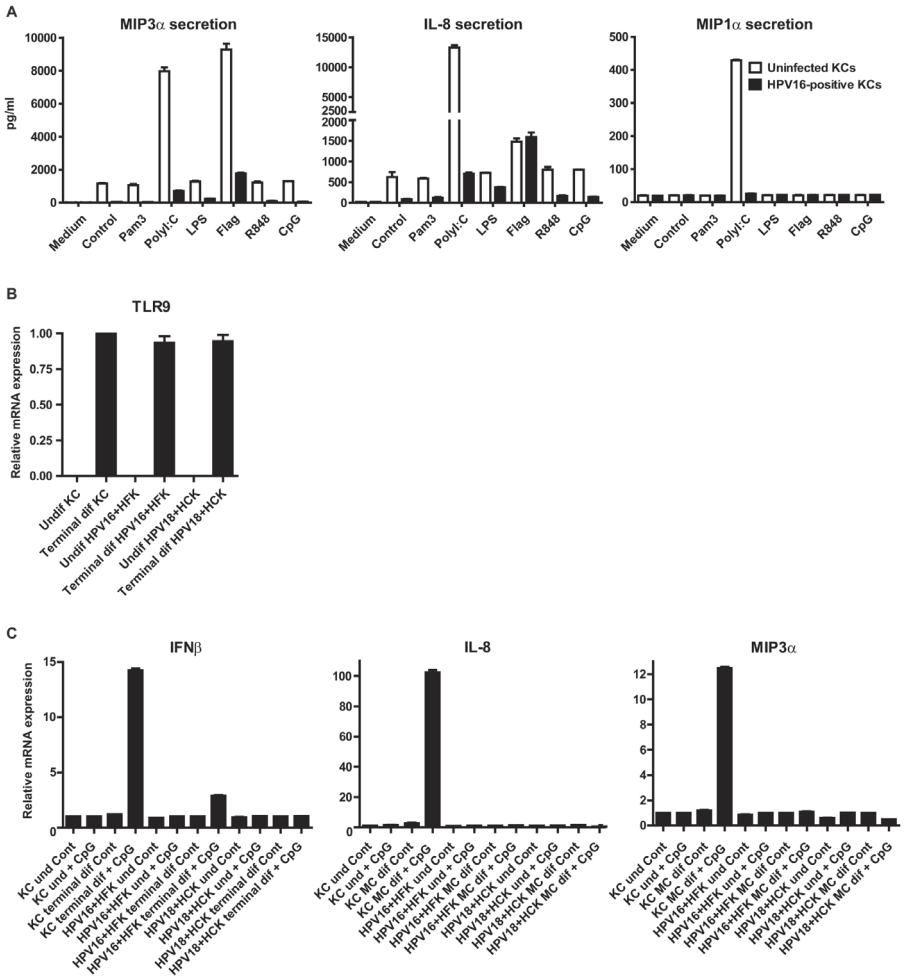

Figure 1. The presence of high risk human papillomavirus interferes with pattern recognition receptor (PRR) signaling of keratinocytes. (**A**) Cytokine production of non-differentiated uninfected or HPV16+ keratinocytes after stimulation with different indicated PRR stimuli as measured by ELISA. (**B**) *TLR9* expression as measured by qRT-PCR on total RNA samples from undifferentiated (und) and terminally differentiated (terminal dif) uninfected KCs, and HPV16 and HPV18 positive KC cultures. (**C**) *IFNβ*, *IL-8* and *MIP3α* expression levels in unstimulated or CpG ODN-stimulated uninfected KCs, and two different HPV (16 or 18) positive KC cultures as examined by qRT-PCR. KCs were either left undifferentiated (und) or terminally differentiated (terminal dif) after which they were stimulated with CpG (10 μg/ml) for 7 hours. (**B**–**C**) Gene expression was normalized using *GAPDH* mRNA expression levels.
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was caused by hrHPV, we infected primary keratinocytes with infectious HPV16 virions (Figure 3A) for 24 hours and then stimulated the non-infected and newly infected KCs with poly(I:C) for another 24 hours after which the levels of *IFNβ*, *RANTES* and *MIP3α* transcripts were measured (Figure 3B). After 24 hours of infection there was a small but discernible increase in the levels of these genes indicating that the keratinocytes initially react to the presence of the virus. However, the levels already dropped at 48 hours post-infection indicating that the virus rapidly exerted its PRR-signaling inhibitory effects. In addition, at the same time

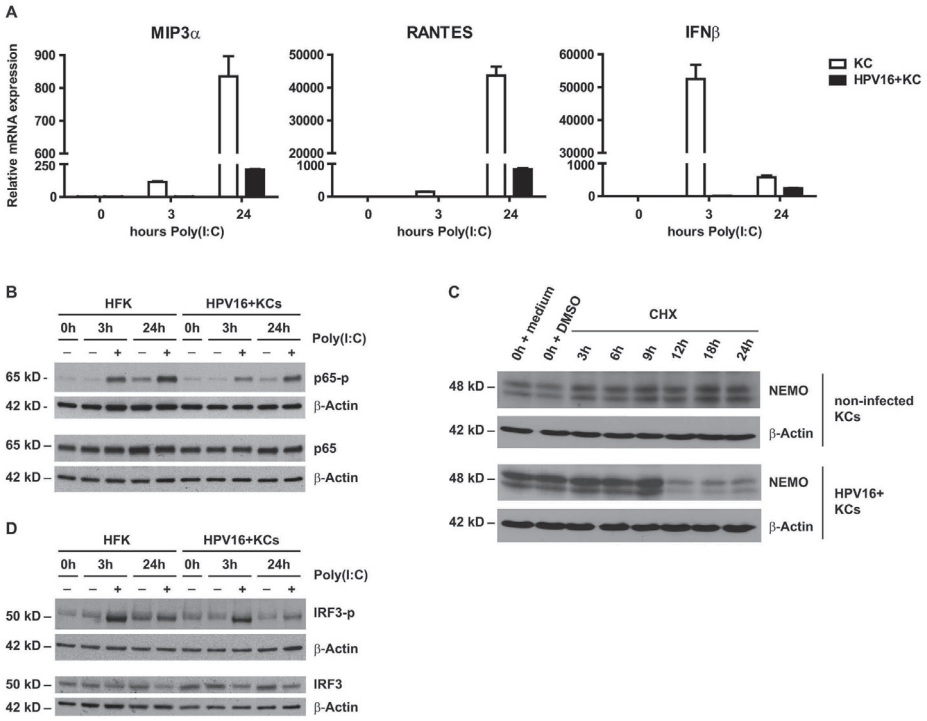

Figure 2. Canonical NF-κB signaling is impaired upstream of the transcription factor p65. (**A**) Poly(I:C) induced cytokine expression in HPV16+ KCs compared to non-infected KCs. *MIP3a, RANTES* and *IFNβ* expression was measured by qRT-PCR. Gene expression was normalized using *GAPDH* mRNA levels and standardized against 0 h of stimulation with poly(I:C). (**B**) Poly(I:C) stimulated phosphorylation levels of p65 in HPV16+ KCs compared to non-infected KCs. Total p65 levels and p65 phosphorylation status were determined in whole cell extracts by western blotting. β-actin served as loading control. (**C**) NEMO degradation in HPV16+ KCs compared to non-infected KCs. Monolayer cultures were treated with 100 μM cycloheximide (CHX) and harvested after 0, 3, 6, 9, 12, 18 and 24 hours. Whole cell extracts were analyzed by western blotting using antibodies against NEMO and β-actin (control for protein degradation). (**D**) Poly(I:C) stimulation-induced phosphorylation levels of IRF3 in hrHPV+ KCs compared to KCs. Total IRF3 levels and IRF3 phosphorylation status were determined in whole cell extracts by western blotting. β-actin served as loading control.

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point these newly hrHPV-infected keratinocytes displayed a hampered activation of *IFNβ*, *RANTES* and *MIP3α* following 24 hours of stimulation with poly(I:C) (Figure 3B). Moreover, we repressed the polycistronic viral mRNA transcript [18,19] in hrHPV+ KCs by the use of siRNA targeting HPV16 *E2* as this allows the destruction of the whole RNA chain. Indeed the suppression of HPV early gene E2 expression translated into an overall decrease in viral early gene expression (Figure 3C) and an increase in the transcription of *IFNβ*, *RANTES* and *MIP3α* following poly(I:C) stimulation (Figure 3D).

Together these data demonstrate that the innate immune response to viral and bacterial-derived PRR stimuli of both undifferentiated and differentiated hrHPV+ keratinocytes is suppressed by HPV at a point downstream of the PRR receptors but upstream of the transcription factors that relay the PRR signals to the nucleus.

The ubiquitin-modifying enzyme UCHL1 is over-expressed in hrHPV-positive keratinocytes and responsible for suppressing the production of type I IFN as well as pro-inflammatory and chemotactic cytokines

Our data suggest that hrHPV+ keratinocytes manifest a generalized inability to respond to stimulation through interference at, or downstream of the cytosolic part of the PRR signaling pathways. We therefore re-analyzed the genome-wide expression profiles (Gene Expression Omnibus accession number GSE21260) of several different uninfected KC cultures and hrHPV+ KC cultures reported previously [10] by Ingenuity Pathways Analysis (IPA) and found a highly significant enrichment of genes belonging to the protein ubiquitination pathway (Table S1; $p = 6.69 \times 10^{-5}$). In this pathway, the gene for the enzyme ubiquitin carboxyl-terminal hydrolase L1 (*UCHL1*) was the most upregulated gene in

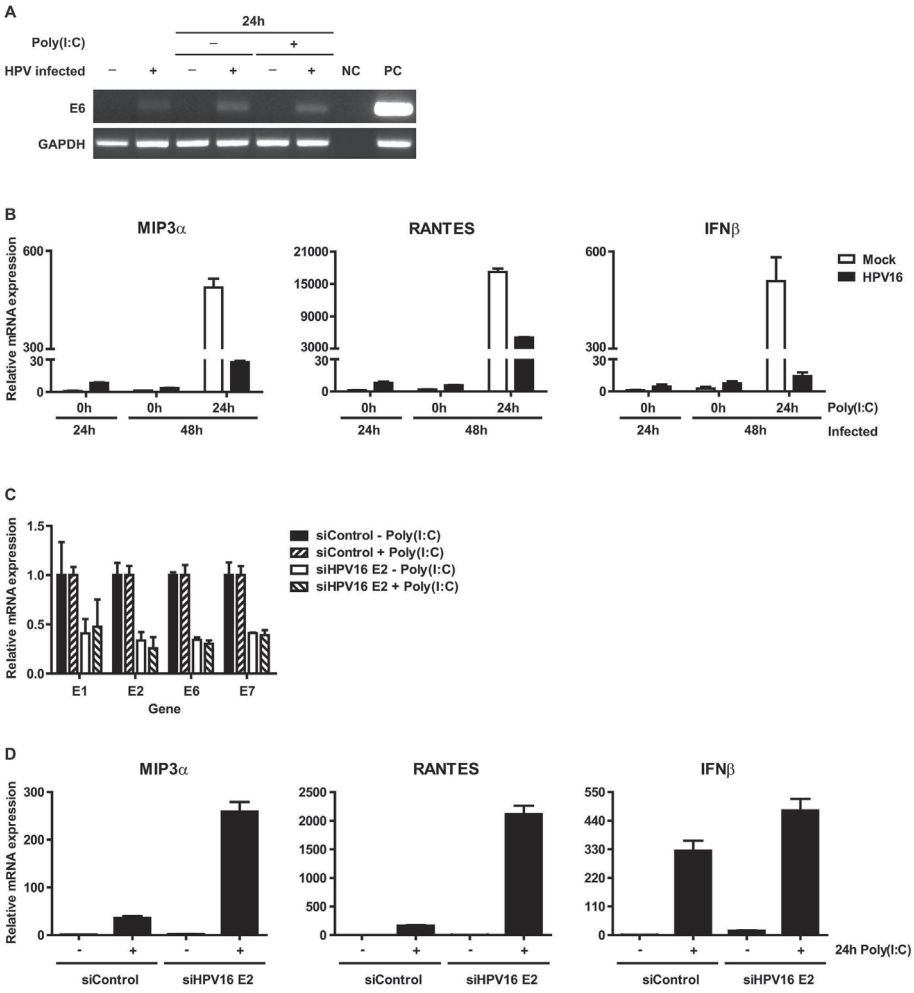

Figure 3. Expression of human papillomaviral transcripts are required to impair cytokine expression of poly(I:C) stimulated keratinocytes. (**A**, **B**) Cytokine expression at the initial stage of HPV16 infection. Primary basal layer human foreskin keratinocytes were infected with native HPV16. (**A**) Viral early gene E6 expression was analyzed 1 and 2 (24 h poly(I:C)) days after infection by PCR. NC: negative control, PC: positive control, HPV16+ KCs. (**B**) *MIP3a*, *RANTES* and *IFNβ* expression was measured by qRT-PCR. Gene expression was normalized against *GAPDH* mRNA levels and standardized against the 0 h poly(I:C) stimulated non-infected cells. Similar results were observed in two independent experiments. (**C**, **D**) Poly(I:C)-induced cytokine expression in HPV+ KCs transfected with control siRNA (siControl) or siRNA targeting HPV16 E2 (siHPV16 E2). *E1*, *E2*, *E6*, *E7* (**C**) as well as *MIP3a*, *RANTES*, and *IFNβ* (**D**) expression was analyzed by qRT-PCR. Gene expression was normalized against *GAPDH* mRNA levels and standardized against no poly(I:C) siControl. For all three genes the response to poly(I:C) was significantly higher when HPV16 E2 was suppressed ($p<0.001$, one-way ANOVA).
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hrHPV+ KCs compared to uninfected KCs (Figure 4A and B). The upregulation of *UCHL1* in hrHPV+ KCs was confirmed by RT-qPCR in both foreskin and vaginal epithelial hrHPV+ KC cultures and expression was not influenced by poly(I:C) activation (Figure 4C). Furthermore, UCHL1 upregulation at the protein level was tested and shown for three different hrHPV+ KCs by

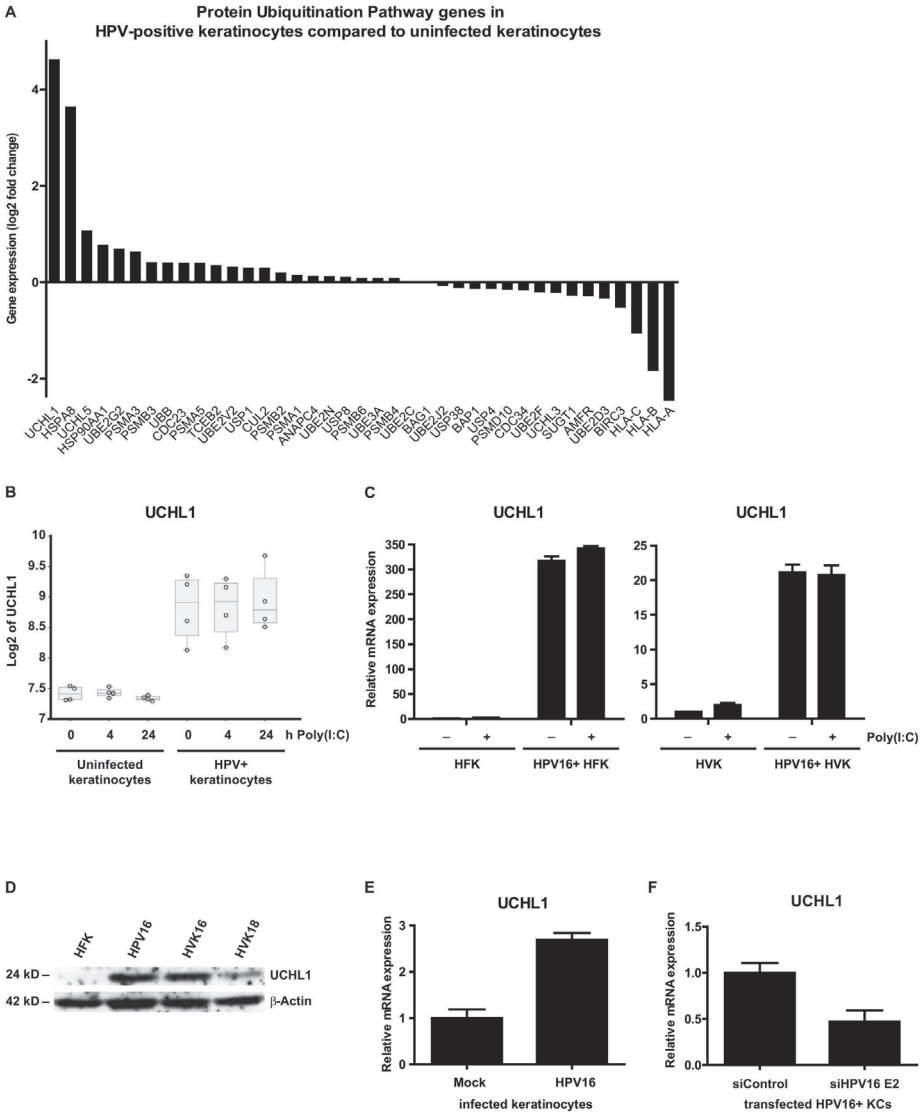

Figure 4. HPV induces expression of *UCHL1* in keratinocytes. (**A**) Summary of all differentially expressed genes within the Protein Ubiquitination Pathway. Differentially expressed genes between four uninfected KC and four hrHPV+ KC cultures with adjusted *p*-value≤0.05 identified 24 hours after poly(I:C) stimulation by microarray analysis (log2 ratios) are shown. (**B**) *UCHL1* microarray gene expression values (log2 intensities) after 0, 4, and 24 hours of poly(I:C) stimulation in four primary KCs and four hrHPV+ KCs (circles). The box represents the 25th and 75th percentiles, the median is indicated with a horizontal line within the box, and the whiskers represent the minimum and maximum. (**C**) *UCHL1* expression in HPV16+ human foreskin keratinocytes (HFK; left panel) and HPV16+ human vaginal keratinocytes (HVK; right panel) when compared to uninfected KCs. KCs were either left unstimulated or stimulated with poly(I:C) for 24 hrs. *UCHL1* expression was normalized against *GAPDH*. (**D**) UCHL1

protein levels in HPV16+ human foreskin keratinocytes (HPV16) and HPV16+ or HPV18+ human vaginal keratinocytes (HVK16 or HVK18, respectively) when compared to non-infected KCs (HFK) as detected by western blotting (WB) in whole cell extracts. β-actin served as loading control. (**E**) *UCHL1* expression at the initial stage of HPV16 infection. Primary basal layer human foreskin keratinocytes were infected with native HPV16 (HPV16 infected keratinocytes) or not (Mock). *UCHL1* mRNA expression was analyzed by qRT-PCR 2 days after infection. Gene expression was normalized against *GAPDH* mRNA levels and standardized against the non-infected cells. Similar results were observed in two independent experiments. (**F**) *UCHL1* expression in HPV+ KCs transfected with control siRNA (siControl) or siRNA targeting HPV16 E2 (siHPV16 E2). *UCHL1* expression was analyzed by qRT-PCR. Gene expression was normalized against *GAPDH* mRNA levels and standardized against siControl. Similar results were observed in more than 3 independent experiments.
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western blotting (Figure 4D). Moreover, expression of *UCHL1* was upregulated 2 days post-infection of HPV16 in primary keratinocytes when compared to mock-infected primary keratinocytes (Figure 4E), whereas knock-down of the polycistronic viral mRNA transcript in hrHPV+ KCs by siRNA for HPV16 E2 resulted in a decreased *UCHL1* expression (Figure 4F). Thus, the cellular deubiquitinase UCHL1 is upregulated by hrHPV.

Although UCHL1 had not been associated with the inhibition of PRR signaling, its enhanced expression in hrHPV+ KCs fits well with the general role of deubiquitinases in controlling PRR signaling [6]. To test whether hrHPV-induced UCHL1 inhibits PRR signaling, we used lentiviral vectors expressing short-hairpin RNA (shRNA) against *UCHL1* and this resulted in a downregulated expression of *UCHL1* transcripts and protein levels in hrHPV+ KCs (Figure 5A and B). Upon stimulation with poly(I:C), hrHPV+ KCs expressing shRNA against *UCHL1* (shUCHL1) but not hrHPV+ KCs expressing a control shRNA (shControl) restored poly(I:C)-mediated induction of type I interferon and proinflammatory cytokines (Figure 5C). Similar results were obtained using transiently transfected RNA interference (RNAi) oligos targeting *UCHL1* but not with control RNAi oligos (Figure S3). An increase in the expression levels of *IL8* and *MIP3α* was detected in hrHPV+ KCs in which *UCHL1* was downregulated. Gene expression increased to the same levels found in *UCHL1*-non silenced hrHPV+ KCs cells stimulated with poly(I:C) (Figure S3). This suggests that downregulation of *UCHL1* increases the gene expression of *IL-8* and *MIP3α* in hrHPV+ KCs. Conversely, transfection of uninfected KCs to overexpress *UCHL1* resulted in a decreased expression of *MIP3α*, *RANTES* and *IFNβ* upon poly(I:C) stimulation (Figure 5D and E). Based on control experiments in which KCs were transfected with green fluorescent protein expressing plasmids, the transfection efficiency of keratinocytes was 30–40% (not shown), indicating that in a large part of the keratinocytes the activation of cytokine-encoding genes is not impaired and explaining the expression levels of these cytokine-encoding genes that are still detected.

All together, these data clearly demonstrate that UCHL1 can downregulate the PRR-mediated activation of both the type I IFN and proinflammatory cytokine and chemokine pathways.

Knock down of UCHL1 increases the phosphorylation of IRF3 and NFκB p65 and alleviates NEMO degradation

We then asked whether the restoration of PRR signaling, as indicated by an increased induction of type I interferon and proinflammatory cytokines by the knock down of UCHL1 in hrHPV+ KCs would also be reflected in the levels of phosphorylated p65 (p65-p) and IRF3 (IRF3-p) upon poly(I:C) stimulation. Therefore, the p65-p and IRF3-p levels were analyzed in whole cell extracts of HPV16+ KCs stably expressing shRNA against *UCHL1* or control shRNA and following 3 h or 24 h of stimulation with poly(I:C). Knock down of UCHL1 in hrHPV+ KCs resulted in increased p65 phosphorylation at 3 and 24 hours after poly(I:C) stimulation (Figure 6A) coinciding with enhanced cyto- and chemokine production (Figure 5C). In addition, analysis of hrHPV+ KCs treated with cycloheximide revealed that NEMO degradation was alleviated when UCHL1 was knocked down by shUCHL1 as compared to the shControl hrHPV+ KCs (Figure 6B). Furthermore, higher levels of phosphorylated IRF3 were detected in hrHPV+ KCs in which *UCHL1* was knocked down as compared to hrHPV+ KCs expressing the shControl after 3 hours of poly(I:C) stimulation (Figure 6C).

UCHL1 alters the poly-ubiquitination of TRAF3 and NEMO

TRAF3 ubiquitination is critical for type I IFN production and is a likely target for ubiquitin-modifying enzymes such as UCHL1. As the biochemical experiments to understand the nature of this interaction would require substantial amounts of primary KCs, which can only grow for a few passages thereby restricting their use in biochemical studies, we switched to the HEK293T cell system that is widely used for these purposes. To investigate the interaction between UCHL1 and TRAF3 we overexpressed UCHL1 and Flag-tagged TRAF3 in HEK293T cells. After FLAG immunoprecipitation, we confirmed that UCHL1 co-immunoprecipitated with TRAF3 (Figure 7A). TRAFs are activated by oligomerization and auto-ubiquitination, a process that results in lysine 63 (K63)-linked poly-ubiquination of TRAF, and this event can be induced by either their overexpression or by receptor activation. In contrast K48-linked poly-ubiquitination results in proteasome-mediated degradation of ubiquitinated TRAFs [6]. To test whether UCHL1 modified TRAF3 ubiquitination status, Flag-tagged TRAF3 and haemagglutinin A (HA)-tagged ubiquitin were overexpressed in control or UCHL1 overexpressing HEK293T cells. Poly-ubiquitination of TRAF3 was clearly visible by immunoblot analysis but strongly reduced when UCHL1 was also overexpressed (Figure 7B, Figure S4). No reduction in poly-ubiquitination was detected when as a control the growth regulated ubiquitin-specific protease 8 (USP8), which similar to UCHL1 displays carboxyl-terminal hydrolase activity, was over-expressed (Figure 7B). The UCHL1-associated decreased detection of poly-ubiquitinated TRAF3 was not the result of increased TRAF3 degradation as blocking the proteasomal degradation pathway by the inhibitor MG132 did not result in a reappearance of poly-ubiquitinated TRAF3 (Figure 7C). Instead, experiments in which HA-tagged ubiquitin mutants 'K63 Only' and 'K48 Only' (where all lysine residues, except at position K63 and K48, respectively, were mutated to arginine) showed that UCHL1 removed K63-linked poly-ubiquitins but not K48-linked poly-ubiquitins (Figure 7D), consistent with the known deubiquitinating capacity of UCHL1 [20]. K63-linked ubiquitination is required for TRAF3 to bind its partner TBK1 to activate the downstream type I IFN-signaling pathway. As expected, UCHL1-mediated deubiquitination of TRAF3 resulted in less TRAF3 bound to TBK1 in UCHL1 overexpressing cells when compared to control cells (Figure 7E). These data clearly show that UCHL1 binds and deubiquitinates TRAF3 resulting in a decreased TRAF3-TBK1 complex formation.

Poly-ubiquitination of TRAF6 and its downstream partner NEMO is critical for the PRR-induced activation of proinflammatory cytokine genes [6]. Since the overexpression of UCHL1

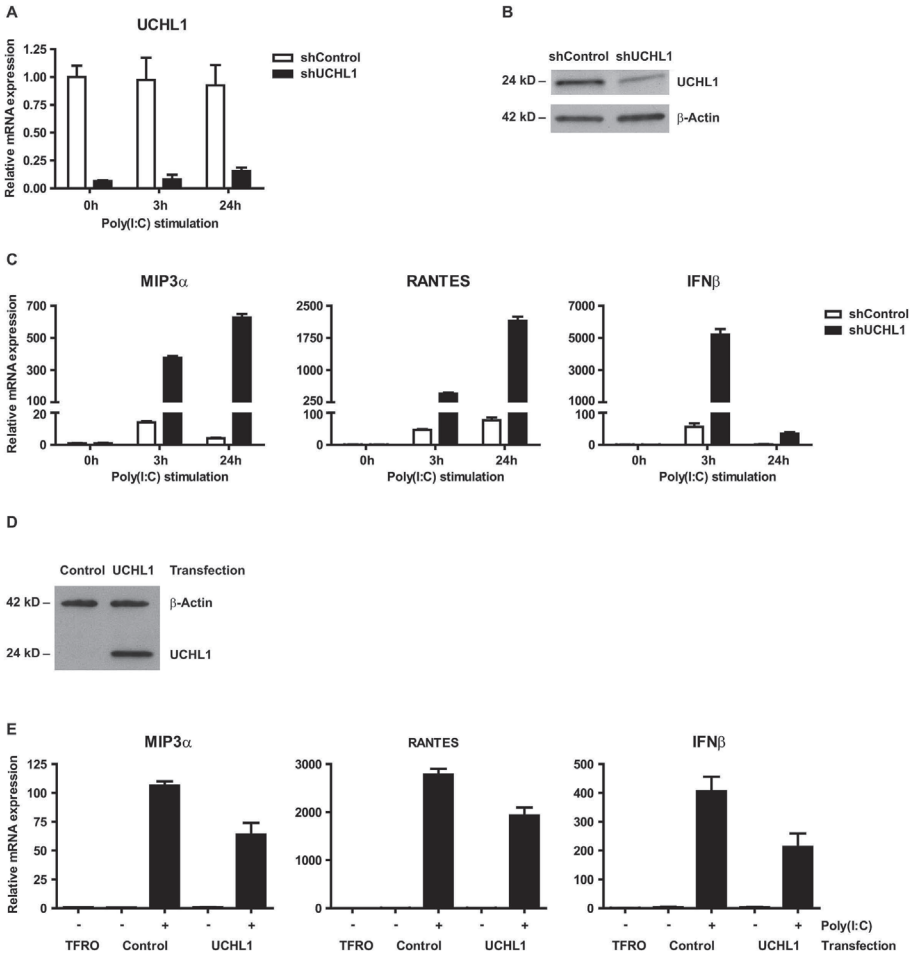

Figure 5. ***UCHL1*** **is responsible for suppressing poly(I:C) mediated gene activation of IFN-I and proinflammatory cytokines in hrHPV-infected KC.** (**A**–**C**) *UCHL1* knock-down effect of poly(I:C) mediated gene expression of IFN-I and proinflammatory cytokines. HPV16+ keratinocytes were transduced with lentiviral vectors expressing shRNA against control mRNA (TurboGFP; shControl) or targeting mRNA of UCHL1 (shUCHL1). Cells were either left unstimulated, or were stimulated with poly(I:C) for 3 or 24 hrs. (**A**) *UCHL1* mRNA expression was analyzed by qRT-PCR and (**B**) UCHL1 protein levels were analyzed by western blotting in whole cell extracts, β-actin served as loading control. (**C**) *MIP3α*, *RANTES* and *IFNβ* mRNA expression was analyzed by qRT-PCR. Gene expression was normalized against *GAPDH* mRNA levels and standardized against 0 h of stimulation with poly(I:C). (**D**, **E**) *UCHL1* overexpression effect on the activation of poly(I:C) mediated gene expression of *IFNβ* and proinflammatory cytokines. Uninfected keratinocytes were transfected with a vector harboring the *UCHL1* gene, an empty control or only received the transfection agent (TFRO). Cells were either left unstimulated, or were stimulated with poly(I:C) for 24 hrs. (**D**) UCHL1 protein levels were upregulated in the *UCHL1*-transfected cells as detected by western blotting in whole cell extracts, β-actin served as loading control. (**E**) *MIP3α* and *RANTES* mRNA expression was analyzed by qRT-PCR. Gene expression was normalized against *GAPDH* mRNA levels and standardized against the TFRO at 0 h of stimulation with poly(I:C).

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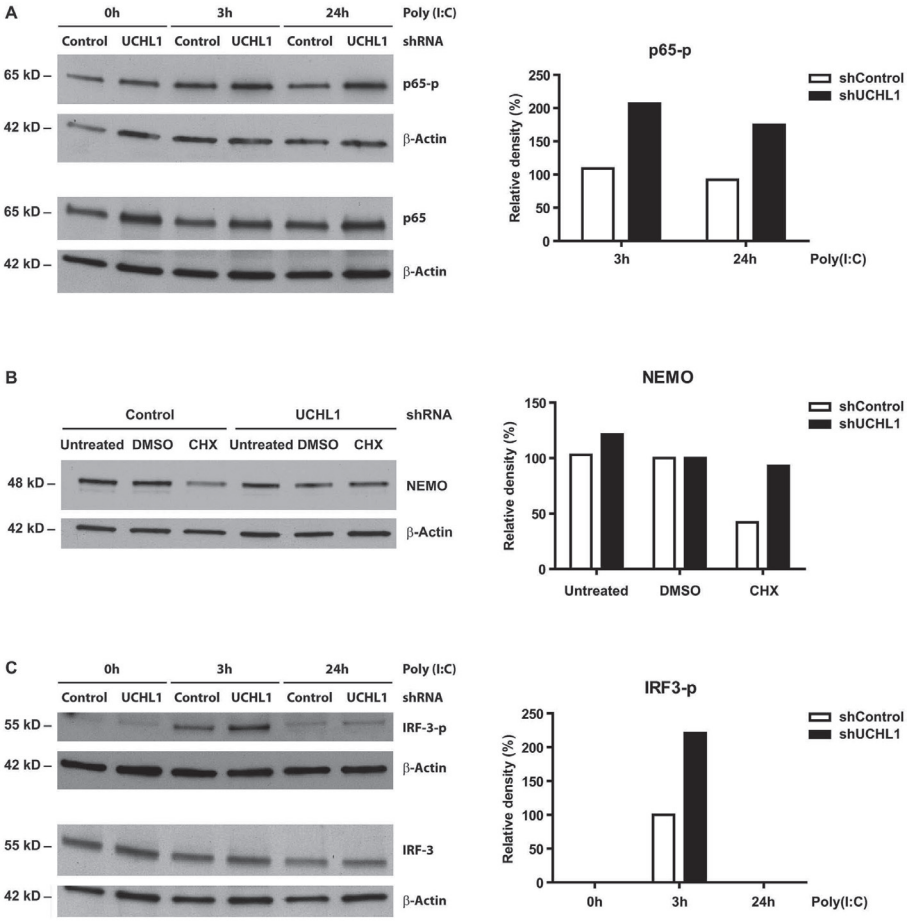

Figure 6. ***UCHL1*** **reduces phosphorylation levels of IRF3 and p65 and degrades NEMO in hrHPV-positive KC.** (**A**) *UCHL1* knock down effect on poly(I:C) stimulated p65 phosphorylation in HPV16+ keratinocytes. Monolayer cultures of shControl or shUCHL1-expressing HPV16+ KCs were stimulated for 0, 3 or 24 hours with Poly(I:C). Whole cell extracts were analyzed by western blotting for p65, p65-p and β-Actin (as loading control). The relative expression of p65-p was quantified by measuring its density and by normalizing it to that of β-Actin. The expression levels of p65-p in the 0 h Poly(I:C) cells were set to 100% for both shControl and shUCHL1 cells. The p65-p levels in the 3 h and 24 h Poly(I:C) cells were calculated against the levels measured at 0 h Poly(I:C) (right panel). (**B**) NEMO protein levels after knock down of *UCHL1* in HPV16+ KCs. Monolayer cultures of shControl or shUCHL1-expressing HPV16+ KCs were treated with 100 μM cycloheximide (CHX) for 16 hours. Whole cell extracts were analyzed by western blot using antibodies against NEMO and β-Actin (control for protein content). The relative expression of NEMO was quantified by measuring its density and by normalizing it to that of β-Actin. The expression of NEMO in the DMSO control was set to 100% (right panel). (**C**) *UCHL1* knock down effect on poly(I:C) stimulated IRF3 phosphorylation in HPV16+ keratinocytes. Similar to A, however cell extracts were analyzed by western blotting using antibodies against IRF3, IRF3-p and β-Actin (as loading control). The relative expression of IRF3-p was quantified by measuring its density and by normalizing it to that of β-Actin. The expression of IRF3-p in the 3 h Poly(I:C) control cells (no knock down of *UCHL1*) was set to 100% (right panel).
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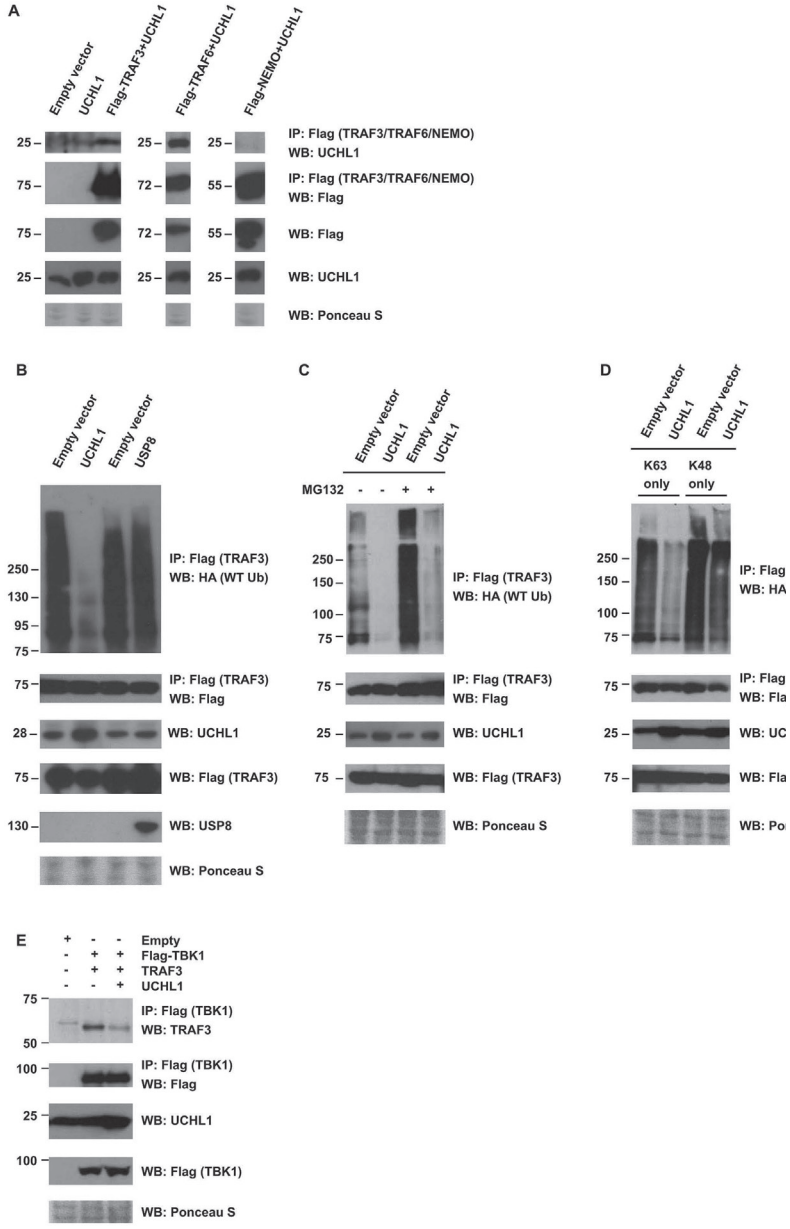
clearly affected proinflammatory cytokine synthesis (Figure 5) the interaction of UCHL1 with TRAF6 and NEMO was tested. Co-expression and immunoprecipitation experiments in HEK293T cells showed that UCHL1 bound to TRAF6 but not to NEMO (Figure 7A). In contrast to what we observed for TRAF3, UCHL1 displayed a modest effect on the poly-ubiquitination of TRAF6


A
Empty vector
UCHL1
Flag-TRAF3+UCHL1
Flag-TRAF6+UCHL1
Flag-NEMO+UCHL1
IP: Flag (TRAF3/TRAF6/NEMO)
WB: UCHL1
IP: Flag (TRAF3/TRAF6/NEMO)
WB: Flag
WB: Flag
WB: UCHL1
WB: Ponceau S
B
Empty vector
UCHL1
Empty vector
USP8
IP: Flag (TRAF3)
WB: HA (WT Ub)
IP: Flag (TRAF3)
WB: Flag
WB: UCHL1
WB: Flag (TRAF3)
WB: USP8
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MG132
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D
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UCHL1
Empty vector
UCHL1
K63 only
K48 only
E
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Flag-TBK1
TRAF3
UCHL1
IP: Flag (TBK1)
WB: TRAF3
IP: Flag (TBK1)
WB: Flag
WB: UCHL1
WB: Flag (TBK1)
WB: Ponceau S

Figure 7. Interaction of UCHL1 with the PRR downstream signaling molecule TRAF3. (**A**) UCHL1 directly interacts with TRAF3 and TRAF6 but not NEMO. HEK293T cells were co-transfected as indicated and the respective TRAF3, TRAF6 or NEMO proteins were immunoprecipitated using Flag antibody, and co-precipitating UCHL1 was detected by WB. As a control a WB analysis for Flag was performed indicating that both TRAF3 and NEMO were present. The bottom three panels show a WB analysis of Flag and UCHL1 of non- immunoprecipitated lysate and a Ponceau S stained loading control for WB. (**B**) UCHL1, but not the control ubiquitin-specific protease 8 (USP8) mediates deubiquitination of TRAF3. HEK293T cells were co-transfected with Flag-TRAF3, HA-tagged wild-type ubiquitin (WT-Ub), and with either empty vector, WT UCHL1 or USP8. TRAF3 was immunoprecipitated with Flag antibody and WB was done with HA or Flag antibodies (top panels). The bottom four panels show a WB analysis of Flag, UCHL1, and USP8 of non- immunoprecipitated lysate and a Ponceau S stained loading control for WB. (**C**) Deubiquitination but not degradation of TRAF3 by UCHL1. HEK293T cells were co-transfected with Flag-TRAF3, HA-tagged wild-type ubiquitin (WT-Ub), and with either empty vector or WT UCHL1. Cells were left untreated or treated with proteasome blocker MG132. TRAF3 was immunoprecipitated with Flag antibody and WB was done with HA or Flag antibodies (top two panels). (**D**) UCHL1 mainly removes K63-linked poly-ubiquitin chains of TRAF3. HEK293T cells expressing Flag-TRAF3, HA-tagged mutant ubiquitin either K63 Only or K48 Only, and WT UCHL1 were immunoprecipitated with Flag antibody and analyzed by HA or Flag antibodies (top two panels). (**E**) UCHL1 lowers TRAF3-TBK1 complex formation. HEK293T cells were co-transfected and TBK1 was immunoprecipitated using Flag antibody, and co-precipitating TRAF3 or TBK1 was detected by WB (top two panels).
doi:10.1371/journal.ppat.1003384.g007

(Figure 8A). However, poly-ubiquitination of NEMO was reduced in UCHL1 overexpressing cells (Figure 8B, Figure S4) but not in USP8 overexpressing cells (Figure 8D). Inhibition of proteasome function by MG132 suggested that the reduced poly-ubiquitination of NEMO was the result of enhanced degradation of NEMO in cells overexpressing UCHL1 (Figure 8C, compare lanes 2 and 4), albeit that the total protein levels of NEMO in these transfected cells remained unaffected. This is not unexpected as also in the endogenous setting (Figures 2 & 6) the degradation of NEMO could only be visualized when the hrHPV+ KCs where pretreated with cycloheximide to prevent new protein synthesis.

Collectively, these data support the notion that UCHL1 can suppress the PRR-signaling pathways necessary for type I IFN and pro-inflammatory cytokine production by the removal of the activating K63 ubiquitins from TRAF3 and the forced degradation of NEMO.

Discussion

We have employed a unique model for hrHPV infection to examine the potential mechanisms underlying the capacity of hrHPV to evade host immunity by suppression of the innate immune response [10]. We utilized primary KC cultures that were newly infected with HPV16 virions or primary KCs stably maintaining the episomal hrHPV genome to show that despite the expression of multiple PRRs the production of IFNβ and pro-inflammatory cytokines and chemokines is suppressed by hrHPV as a consequence of reduced PRR signaling. We provided firm evidence that this suppression depends on the hrHPV-induced upregulation of the cellular ubiquitin-modifying enzyme UCHL1 in infected primary KCs.

Finally, classical biochemical studies in HEK293T cells [11,21,22] performed to understand how UCHL1 mechanistically could suppress the production of type I interferons and pro-inflammatory cytokines revealed that UCHL1 regulated the ubiquitination of the PRR-signaling pathway adaptor molecules TRAF3 and NEMO. UCHL1 removes activating K63-linked ubiquitin molecules from TRAF3 resulting in a lower amount of the downstream signaling complex TRAF3-TBK-1 to suppress the type I IFN pathway. This puts UCHL1 within the family of other deubiquinating enzymes that regulate the PRR pathways by selectively cleaving lysine-63 (K63)-linked ubiquitin chains from TRAFs (e.g. DUBA, OTUB1, OTUB2, A20) [21,22,23,24,25,26]. Furthermore, we showed that UCHL1 bound to TRAF6 and mediated the enhanced degradation of NEMO as a mechanism to suppress the proinflammatory cytokine NF-κB pathway. Notably, the ubiquitin-modifying enzyme A20, a known negative regulator of the TLR pathway, has two ubiquitin-editing domains allowing it to remove and to add ubiquitin chains (22, 26). UCHL1 has also been reported to have these two opposing functions (20). The ligase activity of UCHL1 may explain the ubiquitination of TRAF6 observed in our study. Although UCHL1 did not bind to NEMO, it is known that other deubiquitinating enzymes (*e.g.* CYLD, A20) bind to TRAFs in order to dock on the IKK complex and to associate with NEMO [21,27]. TRAF6-dependent poly-ubiquitination of NEMO is well known [28]. It is highly likely that UCH-L1 acts in a similar fashion and this would fit with TRAF6-NEMO interaction and our observations that NEMO is degraded.

Our data on the suppression of NF-κB signaling via the degradation of NEMO by UCHL1 fits well with earlier observations concerning the overexpression of UCHL1 in vascular cells. Here UCHL1 attenuated TNF-α induced NF-κB signaling and this was associated with stabilization of IκBα and a decrease in its basal ubiquitination [29]. The activation of NF-κB signaling requires IκBα to become degraded following an interaction with the IκB kinase complex (IKK) which comprises NEMO. Hence, the degradation of NEMO may explain previous observations on UCHL1-associated stabilization of IκBα.

UCHL1 is not found to be central in the network of genes affected by hrHPV, suggesting that it is not part of the cellular genes affected in order to assist in HPV genome replication and viral protein production [10]. This indicates that UCHL1 is not directly involved in viral propagation but rather recruited by hrHPV to suppress keratinocyte-mediated production of cytokines and chemokines that would result in the attraction and activation of an adaptive immune response, thereby enabling the virus to persist and propagate.

Many viruses utilize multifunctional viral proteins in order to evade NF-κB- and IRF-mediated immune responses, to favor viral replication and/or to modulate cellular apoptosis and growth pathways [30]. The group of pox viruses have evolved to inhibit NF-κB-signaling by targeting one or more of the many different molecules of this signaling cascade [31]. The vaccinia virus B14 protein is known to inhibit NF-κB signaling by a variety of toll-like receptor agonists at the level of the IKK complex, of which NEMO is a member [32]. The vaccinia virus A64R protein inhibits TRIF-TRAF3-IRF signaling [33]. The pathogenic NY-1 hantavirus Gn protein inhibits TRAF3 signaling by blocking the formation of TBK1-TRAF3 complexes [34] whereas the LMP1 protein of Epstein-Barr virus directly binds to TRAF3 [35]. Furthermore, foot-and-mouth disease virus 3c protease cleaves NEMO [16] and cytomegalovirus M54 protein induces the proteasome-independent degradation of NEMO [17]. In contrast, human papillomaviruses, with a rather limited coding capacity in their genomes, rely for many aspects of their life cycle on the utilization of cellular proteins [36] and this includes the recruitment of different cellular E3 ligases to mediate degradation of cellular proteins through the ubiquitin-proteasomal pathway [37]. UCHL1 is one of the most abundant proteins in the mammalian nervous system and is involved in regulating synaptic

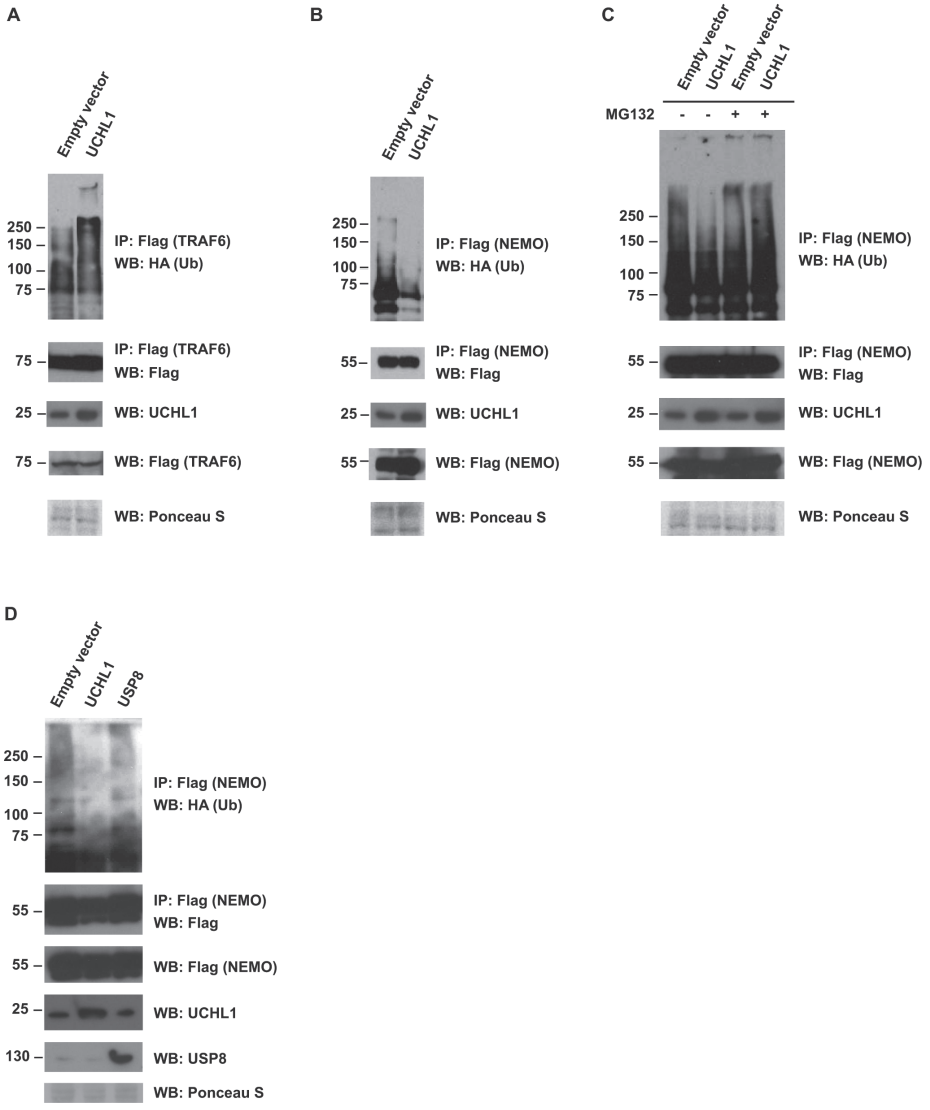

Figure 8. Interaction of UCHL1 with the PRR downstream signaling molecules TRAF6 and NEMO. (**A**) UCHL1 overexpression results in a modest poly-ubiquitination of TRAF6. HEK293T cells were co-transfected with Flag-TRAF6, HA-tagged WT-Ub, and with either empty vector or WT UCHL1. TRAF6 was immunoprecipitated with Flag antibody and western Blotting (WB) was done with HA or Flag antibodies (top two panels). The bottom three panels show a WB analysis of UCHL1 and Flag of non-immunoprecipitated lysate and a Ponceau S stained loading control for WB. (**B**) The effect of UCHL1 on NEMO. HEK293T cells were co-transfected with Flag-NEMO, HA-tagged WT-Ub, and with either empty vector or WT UCHL1. NEMO was immunoprecipitated with Flag antibody and WB was done with HA or Flag antibodies (top two panels). (**C**) The overexpression of UCHL1 mediates the degradation of NEMO. HEK293T cells were co-transfected with Flag-NEMO, HA-tagged WT-Ub, and with either empty vector or WT

UCHL1. Cells were left untreated or were treated with MG132, NEMO was immunoprecipitated with Flag antibody and WB was done with HA or Flag antibodies (top two panels). (**D**) USP8 does not deubiquitinate NEMO. HEK293T cells were co-transfected with Flag-NEMO, HA-tagged wild-type ubiquitin (WT-Ub) and UCHL1 or USP8. NEMO was immunoprecipitated with Flag antibody and WB was done with HA antibodies (top panel). The bottom four panels show a WB analysis of Flag, UCHL1, and USP8 of non-immunoprecipitated lysate and a Ponceau S stained loading control for WB.
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transmission at the neuromuscular junctions [38]. Aberrant expression is related to Parkinson's disease [20] and is also implicated in oncogenesis [39]. In hrHPV+ keratinocytes UCHL1 is expressed and redirected to adopt a new function that is to serve as a negative regulator of the PRR-signaling pathway. As such it mimics the ubiquitin-modifying enzyme A20 which is the natural negative regulator of the TLR pathway [22,26,40]. UCHL1 interferes with the adaptor molecules TRAF3, TRAF6 and NEMO which all function at junctions for the immune stimulating signals from different PRR and type I IFNR to activate NF-κB- and IRF-mediated immune responses. Therefore, the utilization of UCHL1 represents a truly effective use of a cellular protein as it may suppress the immunostimulatory signals initiated through recognition of HPV genomic DNA by TLR9 [5] and RIG-I [11,12] as well as those obtained via the cell surface receptors for type I IFN [41].

The high expression of UCHL1 in primary keratinocytes carrying infectious hrHPV [13,14] is generally lost after transformation of these keratinocytes to tumor cells. Although transformed keratinocytes expressing un-physiologically high levels of *E6* and *E7* via retroviral transduction still may express *UCHL1*, only a minority of spontaneously HPV-transformed cervical carcinoma's and none of the well known HPV-induced cancer cell lines overexpress UCHL1 [42], indicating that under normal conditions *UCHL1* overexpression in HPV transformed cells is not a common event. The expression of the hrHPV oncoproteins E6 and E7 is required to maintain the transformed state of keratinocytes [2,43] suggesting that it is not E6 or E7, but one or more of the other viral proteins responsible for upregulation of UCHL1 (currently under investigation). Previous studies on the innate immune response to hrHPV relied on the overexpression of hrHPV E6 and/or E7 proteins, showing that the viral DNA-sensing TLR9 was altered [8] and that overexpressed HPV E6 or E7 could bind to IRF3 [44] and/or the co-activator CPB [45]. Furthermore, overexpressed hrHPV E6 and/or E7 attenuated IκB kinase signaling [46], and interfered with the nuclear translocation of the interferon-stimulated gene factor 3 (ISGF3) transcription complex [47]. The fact that these studies were performed with only HPV E6- and E7 transfected or transformed cells may explain why the central role of UCHL1 in dampening immunity towards hrHPV+ keratinocytes was not discovered before. In addition, the loss of UCHL1 mediated suppression of the NF-κB pathway in hrHPV E6/E7-induced cancer cells fits well with the notion that solid tumors require the NF-κB-mediated expression of proteins that promote survival, proliferation, invasion and metastasis [48] which is acquired through the E6-mediated deactivation of CYLD [49], a negative regulator of TRAF2 and TRAF6-mediated activation of NF-κB [21,24].

All together, our data implicate UCHL1 as a negative regulator of the PRR pathways helping hrHPV to evade host immunity and allowing it to persist in keratinocytes.

Methods

Cell culture

Primary cultures of human epithelial keratinocytes were established from foreskin [50] and vaginal tissues and grown in serum-free medium (Defined KSFM, Invitrogen, Breda, The Netherlands). Keratinocyte lines stably maintaining the full episomal HPV genome following electroporation were grown in monolayer culture using E medium in the presence of mitomycin C treated J2 3T3 feeder cells [13,14] for two passages and were then adapted to Defined K-SFM for one passage before experimentation. None of the cell cultures were used after passage 15 and the non-transformed state of the cells used was confirmed by the expression of both *E1* and *E2* so that the cells used truly represent the preneoplastic state in which the HPV genomes remained episomal and were capable of the complete viral life cycle. Keratinocytes were terminally differentiated by placing them into serum-free medium containing 1.75% methyl cellulose and 1.8 mM Ca^{2+} for 24 hours [50]. Cells were harvested by washing out the methyl cellulose three times. HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine and 1% penicillin-streptomycin (Gibco-BRL, Invitrogen). Transient transfections were performed using calcium phosphate or Lipofectamine 2000 (Invitrogen).

HPV16 infection of non-infected keratinocytes

Primary basal layer human foreskin keratinocytes were seeded at 7.5×10^4 cells per well of a 24-wells plate in K-SFM and then allowed to attach for 48 hours. Cells received fresh medium (Mock infected) or medium containing native HPV16 isolated from raft cultures at a MOI 100 for 24 hours. Cells were stimulated with or without 25 ug/ml poly(I:C) in K-SFM for 0 or 24 hours and harvested at the indicated time-points.

Plasmid construction

Full length human cDNA clones for UCHL1, TRAF3, TRAF6 and TBK1 were obtained from Open Biosystems (Surrey, UK). The cDNA clones were PCR amplified and subcloned either into pcDNA3.1 expression vector or into Flag-tagged pcDNA3.1 vector. Full-length Flag-NEMO construct was kindly provided by Dr. C. Sasakawa, University of Tokyo, Japan [51]. HA-tagged wild-type and mutant ubiquitin constructs were kindly provided by Dr. A. Iavarone, Columbia University, USA.

RNA expression analyses

Total RNA was isolated using TRIzol (Invitrogen) according to manufacturer's instructions. RNA was purified using RNeasy Mini Protocol (Qiagen, Venlo, The Netherlands). Total RNA (0.2 μg) was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo dT primers (Promega, Madison, USA). TaqMan PCR was performed using TaqMan Universal PCR Master Mix and pre-designed, pre-optimized primers and probe mix for IL-8, MIP-1α, MIP-3α, RANTES, IL-1β, IFNβ, UCHL1 and GAPDH (Applied Biosystems, Foster City, USA). Threshold cycle numbers (Ct) were determined using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and the relative quantities of mRNA per sample were calculated using the ΔΔCt method as described by the manufacturer using GAPDH as the calibrator gene.

Stimulation of cells with TLR ligands and ELISA

5×10^5 cells were plated in 1 ml in each well of 24-well flat bottom plate. Cells were left unstimulated or stimulated with

Pam3CSK4 (5 μg/ml), Poly(I:C) (25 μg/ml), LPS (3.33 μg/ml), flagellin (150 ng/ml), R848 (1 μg/ml), CpG (1 μM) or TNFα (50 ng/ml) for 24 hours. Flagellin was a kind gift from Jean-Claude Sirard (Institut Pasteur, Lille, France). TLR ligands were purchased from Invivogen (San Diego, USA). The supernatants were harvested and IL-8, MIP-3α, and MIP-1α concentrations were determined using corresponding Quantikine ELISA kits (R&D Systems, Oxon, UK).

RNAi and shRNA

Non-targeting RNAi oligos (ON-TARGET*plus* Non-targeting Pool, catalogue D-001810-10-20) and oligos targeting UCHL1 (ON-TARGETplus SMARTpool, catalogue L-004309-00) were purchased from Dharmacon (Chicago, IL). Cells were transfected with RNAi using N-TER Nanoparticle siRNA Transfection System (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions. 24 hours after transfection, cells were stimulated with poly(I:C) (25 μg/ml) for another 24 hours and experiments were performed.

The shRNA's used were obtained from the MISSION TRC-library of Sigma-Aldrich (Zwijndrecht, The Netherlands). The MISSION shRNA clones are sequence-verified shRNA lentiviral plasmids (pLKO.1-puro) provided as frozen bacterial glycerol stocks (Luria Broth, carbenicillin at 100 μg/ml and 10% glycerol) in Escherichia coli for propagation and downstream purification of the shRNA clones. pLKO.1 contains the puromycin selection marker for transient or stable transfection. The construct against UCHL1 (NM_004181) was TRCN0000011079 (LV079): CCGGCAGTTCTGAAACAGTTTCTTTCTCGAGAAAGAAACTGTTTCAGAACTGTTTTT and the control was: SHC004 (MISSION TRC2-pLKO puro TurboGFP shRNA Control vector): CCGGCGTGATCTTCACCGACAAGATCTCGAGATCTT GTCGGTGAAGATCACGTTTTT. HPV16+ KCs were seeded 7.5×10^4 cells per well to a 12-wells plate in K-SFM and were allowed to attach over night. Medium was replaced by infection medium (K-SFM+30% virus supernatant; MOI = 5), containing either the lentivirus LV079 in IMDM 5% FCS or as control SHC004. HPV16+ KCs were infected over night after which infection medium was replaced by K-SFM containing 1000 ng/ml puromycin for 48 hours to select for successfully infected HPV16+ KCs. Then the medium was replaced by K-SFM without puromycin and cells were grown for 24 hours. To stimulate the PRR pathways lentivirus-infected HPV16+ KCs were given K-SFM containing either no poly(I:C) (two wells) or 25 ug/ml poly(I:C) and were cultured for 21 hours. Then one of the two non-stimulated wells received 25 ug/ml poly(I:C) and all cells were cultured for another 3 hours. Cells were harvested and total RNA was isolated.

Silencer Select siRNA against *HPV16 E2* (AACACUACACCCAUAGUACAUtt) was designed using siRNA Target Finder software (Ambion, Invitrogen). Blast search revealed that the designed E2 siRNA does not match with the known human transcriptome. E2 and Negative control #2 (NC2) siRNA (sequence not provided by manufacturer) were purchased from Ambion. HPV16+ KCs were transfected with 50 nM siRNA E2 or NC2 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 48 hours post-transfection cells received K-SFM containing no Poly(I:C) or 25 ug/ml Poly(I:C) and were cultured for 24 hours after which target gene expression was assayed by qRT-PCR.

Western blot analysis and immunoprecipitation

For Western blotting, polypeptides were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a PVDF membrane (Bio-Rad, Veenendaal, The Netherlands). Immunodetection was achieved with anti-Flag (1:2000, Sigma-Aldrich), anti-HA (1:1000, Covance), anti-TRAF3, anti-TRAF6 (both 1:500, Santa Cruz, CA), anti-ubiquitin lysine 48-specific (1:1000, Millipore, Amsterdam, The Netherlands), anti-poly-ubiquitin lysine 63 specific (1:1000, Millipore), anti-TBK1 (1:400, Santa Cruz), anti-NEMO (FL-419, Santa Cruz), anti-UCHL1 (1:1000 Millipore, 1:100 Abcam or 1:1000 Santa Cruz), anti-USP8 (#8728, Cell Signaling Technology, Danvers, MA, USA), anti-phospho-p65 (Ser538; 1:1000, #3033 Cell Signaling Technology) and anti-phospho-IRF3 (Ser396; 1:2000, #4947, Cell Signaling Technology) or β-actin (1:10,000, Sigma-Aldrich) antibodies. The proteins were visualized by a chemoluminescence reagent (Thermo Scientific, Etten-Leur, The Netherlands). X-Ray films were scanned using a GS-800 calibrated densitometer and Quantity One software (Bio-Rad, Veenendaal, The Netherlands) to quantify the intensity of the bands as a measure of the amount of protein of interest in the blot. The relative amount was determined by calculating the ratio of each protein over that of the density measured for the household protein β-Actin.

For immunoprecipitation, cells were collected after 48 h and then lysed in NP40 buffer supplemented with a complete protease inhibitor cocktail (Roche, Almere, The Netherlands). After pre-clearing with protein A/G agarose beads for 1 h at 4°C, whole-cell lysates were used for immunoprecipitation with either mouse or rabbit anti-Flag antibodies (Sigma-Aldrich), or rabbit anti-TRAF3 or rabbit anti-TRAF6. One to two μg of the antibody was added to 1 ml of cell lysate, which was incubated at 4°C for 2–3 h. After addition of protein A/G agarose beads, the incubation was continued for 1 h. Immunoprecipitates were extensively washed with lysis buffer and eluted with SDS loading buffer and boiled for 5 min. For immunoprecipitation under denaturing conditions, proteins were extracted using regular immunoprecipitation buffer plus 1% SDS and heated at 95°C for 5 min. The samples were diluted (10-fold) in regular immunoprecipitation buffer before immunoprecipitation.

Author Contributions

Conceived and designed the experiments: RK BT SA CJMM JMB DG SHvdB. Performed the experiments: RK BT JLB SA VJ JMB. Analyzed the data: RK BT SA CM CJMM JMB DG SHvdB. Contributed reagents/materials/analysis tools: CM CB RO G-JBvO JMB DG SHvdB. Wrote the paper: RK BT RO G-JBvO CJMM JMB DG SHvdB.

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Chapter 4

Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma

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Tumor-Expressed B7-H1 and B7-DC in Relation to PD-1+ T-Cell Infiltration and Survival of Patients with Cervical Carcinoma

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Abstract

Purpose: The interaction between programmed cell death 1 (PD-1), expressed by activated effector or regulatory T cells, and B7-H1 (PD-L1) and B7-DC (PD-L2) results in the inhibition of T-cell function. The aim of this study was to determine B7-H1, B7-DC, and PD-1 expression in cervical carcinoma.

Experimental Design: A tissue microarray of a well-defined group of 115 patients was stained with antibodies against B7-H1 and B7-DC. Three-color fluorescent immunohistochemistry was used to study the number and phenotype of tumor-infiltrating T cells expressing PD-1. Additional analyses consisted of *in vitro* T-cell suppression assays.

Results: B7-H1 was expressed in 19%, and B7-DC was expressed by 29% of the 115 tumors. PD-1 was expressed by more than half of both the infiltrating CD8+ T cells and CD4+Foxp3+ T cells, irrespective of B7-H1 or B7-DC expression by tumors. The expression of B7-H1 did not show a direct impact on patient survival. However, subgroup analysis revealed that patients with a relative excess of infiltrating regulatory T cells displayed a better survival when the tumor was B7-H1 positive ($P = 0.033$). Additional studies showed that the presence of B7-H1 during the activation of CD4+Foxp3+ regulatory T cells impaired their suppressive function in a functional *in vitro* assay.

Conclusions: B7-H1 is expressed on only a minority of cervical cancers and does not influence the survival of patients with cervical cancer. PD-1 is expressed by a vast number of infiltrating CD8 T cells, suggesting that blocking of PD-1 could have therapeutic potential in cervical cancer patients. (Clin Cancer Res 2009;15(20):6341–7)

Cervical cancer is the second most common cancer in women worldwide (1). It develops as a result of an uncontrolled persistent infection with a high-risk type of human papilloma virus (HPV), in particular, types HPV16 and HPV18 (2). The occurrence of HPV-induced cancer is strongly associated with failure to mount a strong HPV-specific type 1 T-helper and cytotoxic T-lymphocyte response (3–5), the lack of CD8+ T cells migrating into the tumor cell nests, the induction of HPV16-specific regulatory T cells, and the influx of regulatory T cells into the tumor (6, 7). Moreover, the ratio between the tumor-infiltrating CD8+ T cells and coinfiltrating CD4+Foxp3+ regulatory T cells is an independent prognostic factor for overall survival (8), indicating the key role of these different types of T cells in cervical cancer.

Activated T cells can express the programmed cell death 1 (PD-1) receptor, which can bind B7-H1 (PD-L1) and B7-DC (PD-L2). B7-H1 could be induced to express by a wide variety of immune cells and nonhematopoetic cell types, whereas B7-DC is expressed mainly on activated macrophages and dendritic cells (9). Upon simultaneous engagement of both, the T-cell receptor and PD-1–negative immunoregulatory signals are transferred to the T cells, resulting in a decreased effector response and T-cell tolerance (10). PD-1/B7-H1 interactions have been shown to inhibit a wide range of immune responses against pathogen, tumor, and self-antigens (11, 12).

More recently, it has been reported that B7-H1 and B7-DC are exploited by tumors to evade immune responses. B7-H1 is found to express on cell surface in most human cancers, and this expression was correlated with poor clinical prognosis in renal, gastric, ovarian, breast, and esophageal carcinomas (13–17). The role of B7-DC in the suppression of immune responses remains controversial (18). Because of the strong association between tumor-infiltrating lymphocytes (TIL) and the prognosis of cervical cancer (6, 8) and the fact that PD-1 has been reported to be expressed by tumor-infiltrating CD8+

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Translational Relevance

The extent of tumor infiltration by T cells and the ratio between the several different subtypes is an independent prognostic factor with respect to the survival of patients with cervical cancer. We have studied B7-H1 and programmed cell death 1 (PD-1) in cervical cancer because the B7-H1–PD-1 axis has been implicated in tumor escape. Our data show that more than half of the tumor-infiltrating CD8+ T cells are positive for PD-1, indicating that these T cells may have become exhausted and die in the event they interact with B7-H1 expressed on tumor cells or antigen-presenting dendritic cells. Given the interest to target PD-1 or B7-H1 for the immunotherapy of cancer our observation bears direct impact on the immunotherapeutic treatment of patients with cervical cancer.

T cells, CD4+ T cells, and regulatory T cells (10), we studied the expression and function of B7-H1, B7-DC, and PD-1 in cervical cancer. Here, we show that PD-1 is expressed on a substantial number of tumor-infiltrating CD8+, CD4+, and regulatory T cells. B7-H1, however, is expressed in only a small group of cervical cancer patients and does not confer a survival disadvantage. Interestingly, when the tumors of this group of patients are infiltrated with a high number of tumor-infiltrating CD4+Foxp3+ regulatory T cells, the expression of B7-H1 may bestow a survival benefit.

Materials and Methods

B7-H1 and B7-DC staining of tissue array. A previously described tissue array containing 115 cervical cancer samples of patients, all of whom underwent a radical hysterectomy, was used for the B7-H1 and B7-DC staining (8). Standard immunohistochemical staining was done using antibodies against human B7-H1 (clone 5H1) and B7-DC (R&D Systems). The tissue array sections were deparaffinized, and antigen retrieval was done using EDTA. To reduce nonspecific binding, sections were incubated overnight at 4°C with 10% rabbit serum. The B7-H1 and B7-DC antibodies were used in 1:200 and 1:800 dilutions, respectively. The Powervision detection system was applied (DAKO). The tissue array was evaluated and scored by two experienced researchers (R. Karim; E.S. Jordanova) independently. Expression groups were defined based on the presence or absence of membranous staining.

Three-color immunostaining for CD8, Foxp3, and PD-1. Eight cases with B7-H1–positive and eight cases with B7-H1–negative cervical cancer specimens were selected from the tissue array based on a comparable amount of tumor-infiltrating T cells to avoid a potential bias between the two groups. The simultaneous immunohistochemical staining of three different epitopes applied to 4-μm, formalin-fixed, paraffin-embedded tissue sections has been reported by us before (6, 8). Briefly, the sections were incubated overnight with a mix of anti-CD8 (4B11; mouse IgG2b; Novo Castra), anti-Foxp3 (clone 236A/E7; Abcam), and anti–PD-1 (R&D Systems) after antigen retrieval with EDTA. Slides were washed and incubated with a combination of the fluorescent antibody conjugates goat anti-mouse IgG2b–Alexa-546, goat anti-rabbit IgG1–Alexa-488, and donkey anti-goat–Alexa-647. Alexa Fluor conjugates were obtained from Molecular Probes. The images were captured with a confocal Laser Scanning Microscope (Zeiss LSM510, Zeiss). Ten images were scanned per slide. For each case, one successive negative control slide was included. The intraepithelial TIL count was presented as the number of cells per square millimeter.

In vitro ***analysis of the effect of B7-H1 on PD-1–positive regulatory T cells.*** A previously isolated HPV16-specific CD4+FoxP3+ regulatory T-cell clone (7) C148.31 and an influenza-specific CD4+Foxp3- T-helper clone (B1.50) were stained with goat anti–PD-1 (R&D Systems), followed by anti-goat biotin (Dako) and streptavidin-allophycocyanin (APC) (eBioscience). PD-1 expression was analyzed by flow cytometry. The effect of B7-H1 on the proliferative response of these two clones was assessed by stimulating 25,000 T cells with 1 μg/mL plate-bound anti-CD3 (OKT-3; Ortho Biotech) and 1 μg/mL plate-bound anti-CD28 (clone L293; BD Biosciences) in the presence or absence of 5 μg/mL plate-bound recombinant human B7-H1/Fc chimera (R&D Systems). The effect of B7-H1 on the regulatory capacity of the CD4+Foxp3+ T-cell clone was tested in a classic suppression assay (7) in which the clone C148.31 was stimulated with 1 μg/mL plate-bound anti-CD3 in the presence or absence of 5 μg/mL plate-bound recombinant human

Table 1. Patient characteristics and relations to B7-H1 and B7-DC expression

Characteristic	Category	*n* (%)	B7-H1, *n* (%)			B7-DC, *n* (%)		
			Negative	Positive	*P*	Negative	Positive	*P*
FIGO stage	Ib1	56 (49)	43 (77)	13 (23)	0.345	45 (82)	10 (18)	0.013
	Ib2/II	59 (51)	50 (85)	9 (15)		35 (59)	24 (41)	
Histopathology	SCC	88 (77)	68 (77)	20 (23)	0.099	67 (76)	21 (24)	0.012
	ADC/ADSC	26 (23)	24 (92)	2 (8)		12 (48)	13 (52)	
Lymph nodes	Negative	84 (74)	65 (77)	19 (23)	0.183	60 (72)	23 (28)	0.488
	Positive	29 (26)	26 (90)	3 (10)		19 (66)	10 (34)	
Tumor size (mm)	<40	66 (57)	55 (83)	11 (17)	0.614	49 (75)	16 (25)	0.276
	≥40	42 (37)	33 (79)	9 (21)		27 (64)	15 (36)	
Vasoinvasion	Negative	69 (64)	54 (78)	15 (22)	0.462	45 (66)	23 (34)	0.279
	Positive	39 (36)	33 (85)	6 (15)		30 (77)	9 (23)	
Infiltration depth (mm)	<15	65 (57)	50 (77)	15 (23)	0.338	45 (70)	19 (30)	1.0
	≥15	49 (43)	42 (86)	7 (14)		35 (71)	14 (29)	
HPV type	HPV16	58 (58)	50 (86)	8 (14)	0.122	42 (74)	15 (26)	0.524
	HPV18	24 (24)	16 (67)	8 (33)		15 (63)	9 (38)	
	Other	17 (17)	14 (82)	3 (18)		13 (76)	4 (24)	

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma; ADC, adenocarcinoma; ADSC, adenosquamous carcinoma.

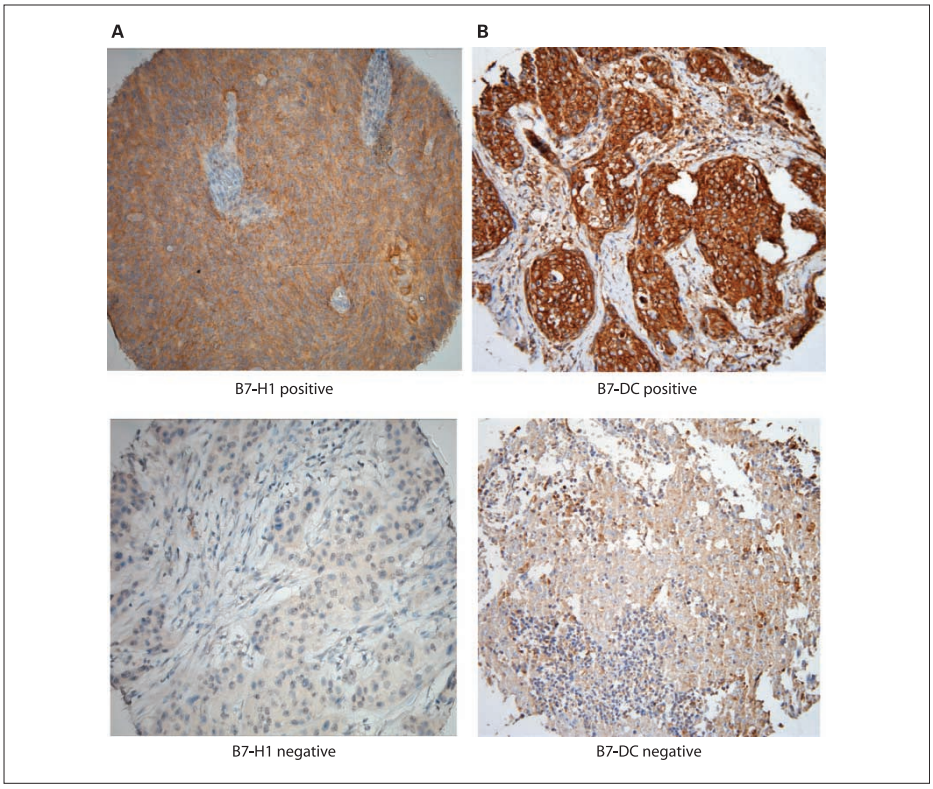

Fig. 1. Immunohistochemical staining of human cervical cancer tissues using B7-H1 and B7-DC antibodies. Expression was defined based on the presence or absence of membranous staining. Specimens with tumor cell surface B7-H1 expression (*A, top*) and tumor with no B7-H1 staining (*A, bottom*), and tumor cell surface B7-DC expression (*B, top*) and tumor with no membranous B7-DC staining (*B, bottom*).

B7-H1/Fc chimera for 24 h. Subsequently, the stimulated C148.31 regulatory T cells were washed to prevent spillover of B7-H1 and put into a coculture with CD4+CD25- responder cells in the presence of 1 μg/mL soluble anti-CD3 and APC (7). After 48 h, the supernatants of triplicate wells were harvested and pooled for the analysis of IFN-γ production by the activated responder cells using enzyme-linked immunosorbent assay (ELISA).

Statistical analyses. Correlations between B7-H1 or B7-DC expression with clinicopathologic parameters or the (high or low) number of tumor-infiltrating cells was done by the χ^2 or, where appropriate, the Fisher's exact test. Patient groups were based on the median (50th percentile) of the numbers of infiltrating immune cells per square millimeter because none of the data for the TIL subtypes followed a normal distribution pattern. Analyses of differences in the numbers of subpopulations of PD-1+ TIL in B7-H1–positive or–negative tumors were done by the nonparametric Mann-Whitney test. All reported *P*s are two sided. A $P < 0.05$ was considered significant. Cumulative 5-y survival rate was calculated by the Kaplan-Meier method and analyzed by the log-rank test. Statistical analyses were done with the SPSS software package 16.

Results

Expression of B7-H1 and B7-DC by cervical cancer cells. To assess the expression and impact of B7-H1 and B7-DC in cervical cancer, we studied a group of 115 well-characterized patients whose clinicopathologic characteristics are shown in Table 1. The mean age of the patients was 48.5 years, with a range between 24 and 87 years at the time of surgery. Fifty-one patients received postoperative radiotherapy because of either tumor-positive lymph nodes or a combination of two of the following parameters: depth of infiltration ≥ 15 mm, tumor size ≥ 40 mm, and presence of vasoinvasion. At the end of the 5-year follow-up period, 23 patients had died of disease, 85 were alive, 5 patients

Table 2. Correlations of B7-H1 and B7-DC with tumor-infiltrating epithelial T cells

Intraepithelial infiltration	Category*	*n* (%)	B7-H1, *n* (%)			B7-DC, *n* (%)		
			Negative	Positive	*P*	Negative	Positive	*P*
CD8+	Low	34 (34)	31 (91)	3 (9)	0.162	23 (68)	11 (32)	0.824
	High	66 (66)	52 (79)	14 (21)		46 (70)	20 (30)	
CD4+	Low	46 (46)	42 (91)	4 (9)	0.060	31 (75)	15 (25)	0.829
	High	54 (54)	41 (76)	13 (24)		38 (70)	16 (30)	
Foxp3+	Low	38 (41)	36 (95)	2 (5)	0.022	27 (71)	11 (29)	1.000
	High	55 (59)	42 (76)	13 (24)		38 (69)	17 (31)	
CD8+/Treg ratio	Low	49 (54)	40 (82)	9 (18)	0.778	33 (67)	16 (33)	0.820
	High	42 (46)	36 (86)	6 (14)		30 (71)	12 (29)	
CD4+/Foxp3 ratio	Low	47 (52)	37 (79)	10 (21)	0.263	30 (64)	17 (36)	0.266
	High	44 (48)	39 (89)	5 (11)		33 (75)	11 (25)	
CD8+/CD4+ ratio	Low	51 (51)	43 (70)	8 (30)	0.794	38 (75)	13 (25)	0.281
	High	49 (49)	40 (82)	9 (18)		31 (63)	18 (37)	

Abbreviation: Treg, regulatory T cell.
*The patients were divided in two categories with low or high numbers of infiltrating cells (or ratio between subtype of cells) based on the 50th percentile.

had a recurrence, and 2 died of causes unrelated to the primary disease but showed no evidence of disease.

The expression of B7-H1 and B7-DC in the tumors of these patients was determined by immunohistochemistry. Specific examples of B7-H1 and B7-DC staining are shown in Fig. 1. Examination of the entire group of 115 patients revealed the expression of B7-H1 in 22 (19%) tumors, whereas B7-DC was expressed in 34 (29%) cases. The expression of B7-H1 and B7-DC did not correlate ($P = 0.604$). Notably, B7-DC expression was associated with cervical adenocarcinomas and a more advanced stage of cervical cancer (Table 1).

B7-H1 and PD-1 expression in relation to the number and type of TILs. The number and subtype of intraepithelial TIL per square millimeter of tumor within this group of 115 patients has already been quantified (8), and this enabled us to analyze the impact of B7-H1 and B7-DC expression on the number and type of intraepithelial TIL in these tumors. The expression of B7-H1 was associated with higher intraepithelial infiltration by Foxp3+ T cells ($P = 0.022$) but not with CD8+ T cells (Table 2). In contrast, there was no significant association between TIL and B7-DC expressed by tumor cells (Table 2).

Negative regulation of the tumor-specific T-cell response by B7-H1 expressing tumor cells requires the intraepithelial infiltrating T cells to express PD-1. Therefore, PD-1 expression by intraepithelial TIL was tested in a group of patients with a B7-H1–positive tumor, as well as in a group of patients with a B7-H1–negative tumor, which were matched with respect to the number of tumor-infiltrating CD8+ T cells, CD4+ T cells, and regulatory T cells (Table 3). In addition, there was no difference in the CD8/Foxp3 ratio ($P = 0.959$) between these two groups, allowing their comparison with respect to PD-1 expression. The number of single-, double- and triple-positive cells for CD8, Foxp3, and PD-1 was analyzed by triple fluorescent immunohistochemistry. This revealed that, in both groups of patients, more than half of the infiltrating CD8+ T cells and half of the Foxp3+ T cells expressed PD-1 (Table 3). Although, on the whole, the patient group with B7-H1–positive tumors displayed somewhat more intraepithelial PD-1+ T cells, this was not significantly different.

B7-H1 expression confers survival benefit in a subgroup of patients with high numbers of intraepithelial infiltrating regulatory T cells. Retrospective analyses of patients with different types of malignancies showed a link between B7-H1 expression on tumors and poor prognosis (13–17). A similar analysis of the overall survival of patients with cervical cancer did not show such a direct relationship ($P = 0.690$; Fig. 2A). Notably, we

Table 3. PD-1 expression on TILs

Cell type	Median cell number per mm^2 (min-max)		
	B7-H1–positive tumor	B7-H1–negative tumor	*P*
CD8+ T cells	198 (48-505)	160 (9.7-314)	0.337
CD4+ T cells	114 (4.8-317)	73 (15-190)	0.170
Foxp3+ T cells	81 (30-219)	53 (16-101)	0.138
CD8+PD-1- T cells	47.5 (7.5-439)	61 (6.1-101)	0.529
CD8+PD-1+ T cells	107.2 (14-252)	85 (1.2-208)	0.462
CD8+Foxp3+ T cells	3.7 (1.1-5.8)	2.3 (0.0-11)	0.713
CD8+PD-1+Foxp3+ T cells	2.2 (0.0-24)	2.4 (0.0-7.1)	0.815
CD4+PD-1+ T cells	56.1 (2.4-193)	16.2 (2.1-138)	0.248
CD4+Foxp3+ T cells	47.0 (0.0-169)	42.2 (5.8-78)	0.345
CD4+PD-1+Foxp3+ T cells	25.3 (2.4-47)	7.7 (3.7-20)	0.074

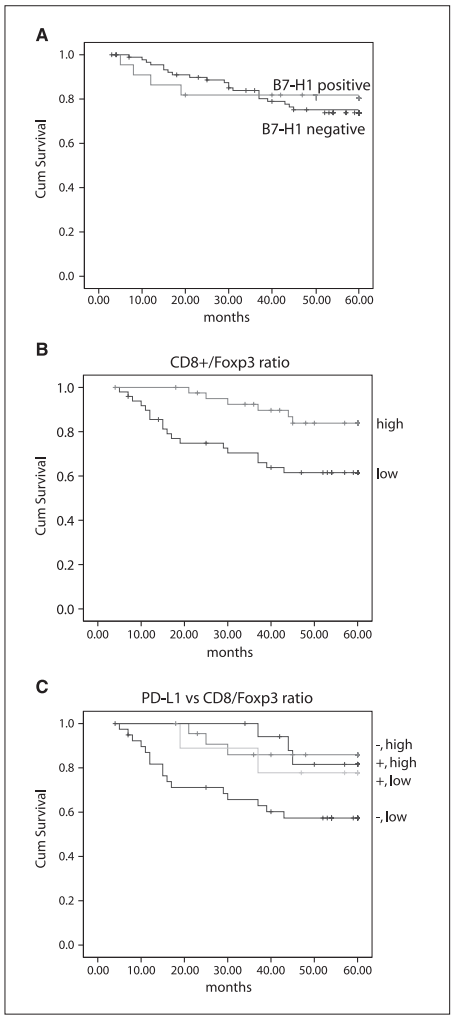

Fig. 2. Kaplan-Meier curves and log-rank test results of 5-y overall survival analyses of patients with cervical cancer based on the expression of B7-H1 on the tumor cell (*A*). Log-rank test result: $P = 0.690$, the ratio between CD8+ and regulatory T cells and the expression of tumor cell surface B7-H1 (PD-L1; *B*) and the ratio between CD8+ and regulatory T cells (*C*). Groups are divided into low (lower 50th percentile) and high (top 50%).

recently reported that the ratio between intraepithelial CD8+ T cells and regulatory (Foxp3+) T cells is an independent prognostic factor for survival in this group of patients with cervical cancer, in which patients with a low CD8/regulatory T cell ratio had the worst survival (ref. 8; Fig. 2B). As a consequence, a potential detrimental effect of B7-H1 expression on survival is more likely to be observed within patients with well-infiltrated tumors. No effect of B7-H1 was seen in the subgroups divided either on the basis of CD8+ T-cell infiltration ($P = 0.584$) or on the number of regulatory T cells ($P = 0.100$; data not shown). In contrast, subdivision based on the CD8+/regulatory T-cell ratio revealed that the overall survival of patients with a B7-H1–positive tumor and a low CD8+/regulatory T-cell ratio was significantly better than in patients with a B7-H1–negative tumor and a low CD8+/regulatory T-cell ratio ($P = 0.033$; Fig. 2C). These data indicate that B7-H1 expression does not have a direct detrimental effect on the overall survival of patients with cervical cancer but on the contrary may improve survival of a small subgroup of cervical cancer patients with tumors relatively heavily infiltrated by regulatory T cells.

PD-1 expression in regulatory T cells and effect of B7-H1 ligation on PD-1–positive regulatory T cells. The association of B7-H1 expression by tumor cells and the enhanced survival of a small subgroup of patients with a low CD8+/regulatory tumor-infiltrating T-cell ratio (Fig. 2C) suggest that the function of regulatory T cells is affected by B7-H1. To test this hypothesis, we made use of a HPV16-specific CD4+Foxp3+ regulatory T-cell clone, which we had isolated from a HPV16+ patient with cervical cancer (7) and which expressed PD-1 at its surface (Fig. 3A), similar to what we observed *in situ* in cervical cancer (Fig. 3B). To determine if B7-H1 ligation has an effect on PD-1 expressing T cells, this CD4+Foxp3+PD-1+ regulatory T-cell clone was stimulated with anti-CD3 in the presence or absence of recombinant B7-H1 protein to test its proliferative capacity. Whereas the proliferation of a PD-1–negative helper T-cell clone was not affected, the PD-1–positive regulatory T-cell clone proliferated less well when B7-H1 was present in the culture (Fig. 3C). To assess whether B7-H1 ligation also inhibited the suppressive capacity of PD-1+ regulatory T cells, the HPV16-specific CD4+Foxp3+PD-1–positive regulatory T cells were stimulated with anti-CD3 in the presence or absence of recombinant B7-H1 protein and then cocultured with CD4+CD25- responder cells (Fig. 3D). Responder cells alone produced high amounts of IFN-γ, but this capacity was suppressed when activated regulatory T cells were added to the culture in a dose-dependent fashion. The presence of recombinant B7-H1 during the activation of the regulatory T cells had a clear negative impact on their suppressive capacity because the IFN-γ production by the responder cells was partly restored (Fig. 3D). These data show that, in principle, PD-1+ regulatory T cells can be incapacitated with respect to their suppressive function when engaged by B7-H1.

Discussion

Following the initial reports that tumor-associated B7-H1 could serve as an immune escape mechanism in a mouse tumor model through downregulation of tumor-specific T-cell responses (19, 20), several retrospective studies in human cancer cohorts showed that B7-H1 expression was associated with clinicopathologic markers for poor prognosis (13, 17) or with lower overall survival (14–16). Our study indicates that most (80%) cervical cancers are B7-H1 negative and that the survival of B7-H1–positive cases is not negatively affected (Figs. 1 and 2). This suggests that tumor-expressed B7-H1 does not play a

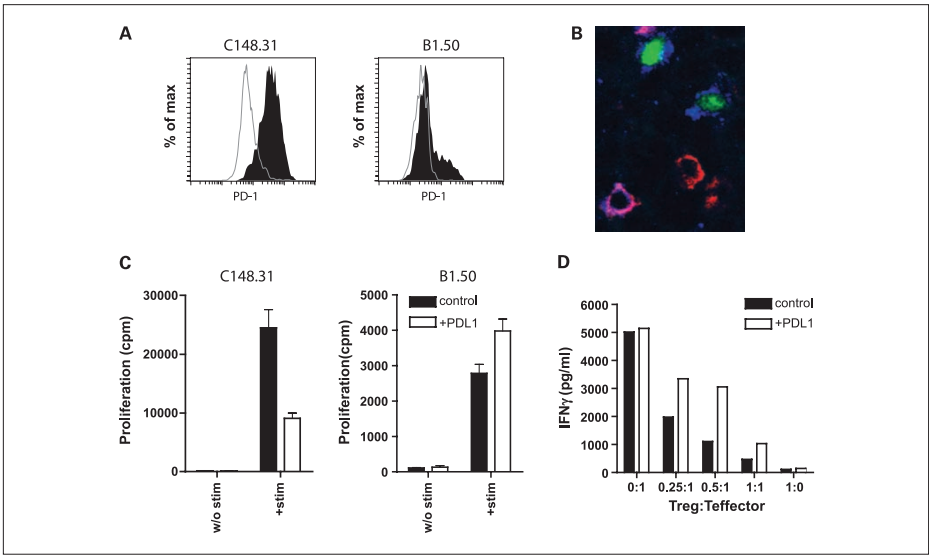

Fig. 3. HPV-specific FoxP3+ regulatory T-cell clone expresses PD-1 and is functionally impaired upon PD-1 ligation. *A*, PD-1 expression (*black areas*) and isotype controls (*gray lines*) of HPV-specific FOXP3+ clone C148.31 and an influenza-specific helper T-cell control clone B1.50. *B*, PD-1 expression observed *in situ* in intraepithelial Foxp3+ T cells in cervical cancer. The different intraepithelial T cells are depicted as CD8+ cells (*red*), PD-1+ cells (*blue*), and Foxp3+ cells (*green*). *C*, proliferation of C148.31 and control clone upon stimulation with plate-bound anti-CD3 and anti-CD28 in the presence or absence of plate-bound B7-H1. *D*, classic suppression assay in which the capacity of the C148.31 regulatory clone to suppress the IFN-γ production of CD4+CD25- cells is tested. C148.31 was pretreated either with plate-bound anti-CD3 and B7-H1 or with plate-bound anti-CD3 only.

significant role in T-cell antitumor immune response in cervical cancer. Rather, our data show that more than half of the tumor-infiltrating CD8+T cells are positive for PD-1, which has previously been shown to be indicative for chronic antigen stimulation and T-cell exhaustion (9).

Although studies reported a negative impact of B7-H1 on the overall survival of patients with either renal or esophageal cancer (15, 16), we did not observe such a direct link between B7-H1 and survival in cervical cancer. A direct effect of B7-H1 on survival likely requires that this survival is associated with CD8+ T-cell infiltration and that these CD8+ T cells express PD-1. Indeed, in esophageal cancer, the infiltration with CD8+ T cells is an independent prognostic factor (21). It will be of interest to determine the proportion of PD-1 expressing CD8+ T cells in renal and esophageal cancer for a better assessment of their role in the observed negative association between B7-H1 and survival in these cancers. The fact that, in cervical cancer, the ratio between CD8+ T cells and coinfiltrating regulatory T cells functions as an independent prognostic factor (8) suggests that the interplay between the two cell types and, as such, the regulation of both cell types is important for survival in cervical cancer. In this view, we made an interesting observation that, among cervical cancer patients whose tumors were infiltrated with relatively higher number of regulatory T cells (low CD8:Foxp3 ratio), tumor-expressed B7-H1 may have conferred a survival benefit (Fig. 2C). Although the underlying mechanism remains to be elucidated, our data provide evidence that it may involve the functional impairment of regulatory T cells because a substantial portion of these cells express the receptor PD-1 *in situ* (Table 3, Fig. 3) and engagement of this receptor through B7-H1 decreases the capacity of an HPV16-specific CD4+Foxp3+ regulatory T-cell clone to suppress the function of effector cells *in vitro* (Fig. 3D). The absence or presence of B7-H1 in the group of patients with a high CD8+/regulatory T-cell ratio did not affect the overall survival (Fig. 2B), suggesting either that B7-H1 does not play a role in patients with a high number of tumor-infiltrating CD8+ T cells and a low number of regulatory T cells or that the stimulatory interaction between B7-H1 and B7.1 on CD8+PD-1- T cells (22) balances the negative interaction between B7-H1 and PD-1+ CD8 T cells within the local tumor environment. It seems, therefore, that B7-H1–mediated impairment of PD-1+ T cells particularly affects the PD-1–expressing regulatory T cells, thereby releasing the brake on the tumor-specific CD8+ T cells. Future experiments using primary tumor-infiltrating T-cell cultures may shed more light on this. Recent studies support the notion that PD-1 expression on regulatory T cells is associated with improved survival (22) and B7-H1–mediated inhibition of proliferation and function of regulatory T cells (23).

From a clinical standpoint, this study represents an unselected series of patients. Given the interest to target PD-1 or B7-H1 for the immunotherapy of cancer, our study suggests that

treatment with PD-1– or B7-H1–blocking antibodies is a viable option. Although we have studied PD-1 expression in patients with stage IB or stage II disease, PD-1– or B7-H1–targeted immunotherapy is likely to be applied in a more advanced stage of cervical cancer. Although we speculate that PD-1 expression will be even more pronounced in advanced stage of cervical cancer, it will be of interest to determine PD-1 expression on T cells at this stage too. The PD-1–B7-H1 pathway also plays an important role in dendritic cell–T-cell interactions (9). One can easily envisage that B7-H1+ dendritic cell, cross-presenting tumor antigen (e.g., E6 and E7 of HPV), may impair the function of responding PD-1+ tumor–specific T cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Chapter 5

CXCR7 expression is associated with disease-free and disease-specific survival in cervical cancer patients

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CXCR7 expression is associated with disease-free and disease-specific survival in cervical cancer patients

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BACKGROUND: The CXC chemokine receptor (CXCR)7 is involved in tumour development and metastases formation. The aim of the present study was to determine protein expression of CXCR7, its putative co-receptors epidermal growth factor receptor (EGFR) and CXCR4, its predominant ligand CXCL12, their co-dependency and their association with survival in cervical cancer patients.

METHODS: CXC chemokine receptor 7, EGFR, CXCR4 and CXCL12 expression were determined immunohistochemically in 103 paraffin-embedded, cervical cancers. Subsequently, associations with patient characteristics were assessed and survival analyses were performed.

RESULTS: CXC chemokine receptor 7 was expressed by 43% of tumour specimens, in a large majority of cases together with either EGFR or CXCR4 (double positive), or both (triple positive). The CXCR7 expression was associated with tumour size ($P = 0.013$), lymph node metastasis ($P = 0.001$) and EGFR expression ($P = 0.009$). CXC chemokine receptor 7 was independently associated with disease-free survival (hazard ratio (HR) = 4.3, 95% confidence intervals (CI) 1.7–11.0, $P = 0.002$), and strongly associated with disease-specific survival (HR = 3.9, 95% CI 1.5–10.2, $P = 0.005$).

CONCLUSION: CXC chemokine receptor 7 expression predicts poor disease-free and disease-specific survival in cervical cancer patients, and might be a promising new therapeutic marker. In a large majority of cases, CXCR7 is co-expressed with CXCR4 and/or EGFR, supporting the hypothesis that these receptors assist in CXCR7 signal transduction.

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Keywords: cervical cancer; CXCL12; CXCR4; CXCR7; EGFR; survival

Cervical cancer is the third most common type of cancer among women worldwide, accounting for 9% of all new cancer cases among females in 2008 (Jemal *et al*, 2011). Although screening for premalignant stages of cervical cancer and vaccination with the available human papillomavirus (HPV)-vaccines are good options to prevent cervical carcinogenesis, treatment options are limited when women present with advanced cancer stages. Partly, this is because the mechanisms involved in tumour cell invasion and metastasis formation, which strongly predict mortality rates, are not fully understood. Furthermore, markers that accurately predict response to therapy are limited. Additional predictive molecular markers for lymph node metastases and disease-free survival may help to elucidate these mechanisms.

A promising new marker is the CXC chemokine receptor (CXCR) 7. CXC chemokine receptor 7 is transcribed from the *RDC1*-gene on chromosome 2, where the genes encoding CXCR1, CXCR2 and CXCR4 are also localised (Balabanian *et al*, 2005). CXC chemokine receptor 7 is strongly expressed in many different tumour types and on tumour-associated vasculature, whereas expression in most normal tissues is weak or absent. The CXCR7 is involved in cell survival, cell adhesion, tumour development and metastases formation (Burns *et al*, 2006; Miao *et al*, 2007). Expression of CXCR7 has not been determined in cervical cancer, but has been associated with a higher tumour grade and more aggressive tumour growth in other cancer types, such as prostate cancer, non-small cell lung cancer, breast cancer, glioma and hepatocellular carcinoma (Miao *et al*, 2007; Wang *et al*, 2008; Iwakiri *et al*, 2009; Hattermann *et al*, 2010; Zheng *et al*, 2010). However, most studies have been performed on tumour cell lines and mouse models, and studies addressing the association between CXCR7 expression and prognosis in patient-derived tumour material are limited.

Two ligands bind to CXCR7, namely CXCL11 (interferon-inducible T-cell α chemoattractant) and CXCL12 (stromal cell-derived factor-1). In cell lines, activation of CXCR7 by CXCL12 induced *trans*-endothelial migration, whereas CXCR7 antagonists and CXCL11 both inhibited cellular migration (Zabel *et al*, 2011). Furthermore, CXCL12 expression was associated with overall and disease-free survival in pancreas carcinoma, suggesting that CXCL12 is the predominant ligand associated with CXCR7-mediated metastasis formation (Liang *et al*, 2010).

Conflicting results have been published on the intracellular signalling pathways of CXCR7, as ligand binding does not result in typical CXC receptor Gi-mediated signalling (Thelen and Thelen, 2008). It is still unclear whether the receptor is able to activate

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other signal transduction pathways. It has been proposed that CXCR7 heterodimerisation with other signalling receptors such as the epidermal growth factor receptor (EGFR) or CXCR4 is required to induce intracellular signalling (Hartmann *et al*, 2008; Décaillot *et al*, 2011; Singh and Lokeshwar, 2011). Both EGFR and CXCR4 expression have been shown to be associated with lymph node metastases and disease-free survival in cervical cancer (Kersemaekers *et al*, 1999; Kodama *et al*, 2007; Schrevel *et al*, 2011).

The aim of the present study was to determine protein expression of CXCR7, its putative co-receptors EGFR and CXCR4, its main ligand CXCL12, their co-dependency and their association with survival in cervical cancer patients. Therefore, we immunohistochemically determined CXCR7, EGFR, CXCR4 and CXCL12 expression, and assessed the association between protein expression and patient characteristics. Furthermore, we analysed whether (co-)expression of CXCR7, EGFR and CXCR4 was associated with disease-specific and disease-free survival.

MATERIALS AND METHODS

Subjects

Formalin-fixed, paraffin-embedded primary tumour tissue samples from 103 cervical cancer patients who underwent radical hysterectomy with lymphadenectomy between January 1985 and December 1999 were collected from the archives of the Department of Pathology, Leiden University Medical Center (Leiden, the Netherlands). Patients had not received radiotherapy or chemotherapy before surgery. Postoperative radiotherapy was indicated in patients with lymph node metastasis, parametrial involvement or positive resection margins. Clinical and follow-up data were taken from patient medical records. Disease-specific survival and disease-free survival were assessed to determine the time to cancer-related death and disease recurrence, respectively. Disease-specific survival time was assessed from the date of surgery to the date of cancer-related death or the date of the last follow-up visit for censored observations. For disease-specific survival, patients who died of a cause unrelated to cervical cancer were considered as censored observations at the date of death. The end point for disease-free survival was the date of local or regional recurrence or the date of distant metastasis.

Tumours were HPV-typed by general primer PCR and sequencing, as described by Koopman *et al* (1999). In addition to the tumour samples, two specimens of normal cervical epithelium were obtained from two patients with no history of cervical cancer. This number of healthy tissue samples was sufficient, as the staining of CXCR4, CXCR7 and CXCL12 was expected to be similar for all normal cervical epithelium specimens. Tissue samples were used according to the guidelines of the Ethical Committee of the Leiden University Medical Center.

Immunohistochemistry

For immunohistochemical analysis, 4 μm tissue microarray (TMA) slides consisting of triplicate punches of 103 cervical cancer patients were deparaffinised and rehydrated. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide (H_2O_2) for 20 min. Antigen retrieval was performed in 0.01 M citrate buffer (pH = 6.0, 12 min, microwave oven). Subsequently, slides were incubated overnight at room temperature with anti-CXCR4 (1 : 600, IgG2a, clone 2F1, Abnova, Heidelberg, Germany), anti-CXCR7 (1 : 50, IgG1, clone 11G8, R&D Systems, Abingdon, UK) or anti-CXCL12 (1 : 50, IgG1, clone 79018, R&D Systems), diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. After washing with PBS, the TMA slides were incubated for 30 min with BrightVision-Poly/HRP (Immunologic, Duiven, the Netherlands). Immunoreactions were visualised using 0.5% 3,3′-diamino-benzidine-tetra-hydrochloride and 0.002% H_2O_2 in Tris-HCl, after which the slides were counterstained with haematoxylin. Immunoreactivity was scored as negative, weak, moderate or strong staining intensity. Immunohistochemistry for EGFR was performed as previously described, and EGFR was scored for membrane staining intensity (Schrevel *et al*, 2011).

Statistical analyses

Statistical analyses were performed using the SPSS program (Version 17.0 for Windows; SPSS Inc., Chicago, IL, USA). Significance tests were two-sided and statistical significance was assumed when $P < 0.05$, corresponding to 95% confidence intervals (CI). To assess whether CXCR7 expression was associated with expression of its ligand CXCL12 or its proposed co-receptors EGFR and CXCR4, the Spearman's rank correlation test was performed, as the intensity scores for CXCR7, EGFR, CXCR4 and CXCL12 were ordinal. The χ^2 test was used to determine whether CXCR7, EGFR, CXCR4 and CXCL12 expression was associated with clinicopathological characteristics, with protein expression data recoded into binary variables. Univariate Cox-regression analysis was performed to assess the association between clinicopathological parameters, CXCR7, EGFR, CXCR4 and CXCL12 expression, and disease-free survival. All analyses were repeated for disease-specific survival. Multivariate Cox-regression analysis was performed to determine whether CXCR7 expression was independently associated with disease-free survival, when correcting for HPV-type, histopathological diagnosis, tumour size, infiltration depth, parametrial invasion, vasoinvasion, lymph node metastasis, resection margins and postoperative radiotherapy. Corresponding survival curves were estimated by the Kaplan-Meier method.

RESULTS

Patient characteristics in relation to CXCR7, EGFR, CXCR4 and CXCL12 expression in cervical cancer

Immunohistochemical staining of CXCR7, CXCR4 and CXCL12 was observed to be both cytoplasmic and membranous in cervical cancer specimens. Normal cervical epithelium stained weakly for CXCR7, CXCR4 and CXCL12, with strong CXCL12 expression in cells of the basal layer. Representative examples of positive and negative CXCR7, EGFR, CXCR4 and CXCL12 cervical cancer specimens are shown in Figure 1. The distribution of staining intensities is shown in Table 1. CXC chemokine receptor 7, EGFR and CXCL12 had a wider range in staining intensity, when compared with CXCR4, as no strong positives were observed for CXCR4. To assess whether CXCR7 expression was associated with expression of its ligand CXCL12 or its proposed co-receptors EGFR and CXCR4, the Spearman's rank correlation coefficient (ρ; rho) was determined. CXC chemokine receptor 7 expression was significantly correlated with EGFR expression ($\rho = 0.272$, $P = 0.009$), but not with CXCR4 expression ($\rho = 0.117$, $P = 0.259$) or CXCL12 expression ($\rho = -0.028$, $P = 0.790$). Compared with an ideal correlation of 1, the observed Spearman's ρ of 0.272 indicates a moderate correlation.

Clinicopathological characteristics of the 103 cervical cancer patients are summarised in Table 2. The median age at the time of diagnosis was 48 years (range, 24–87). Positive CXCR7 expression was associated with tumour size $\geqslant$40 mm (odds ratio (OR) = 2.9, 95% confidence interval (CI) 1.2–6.8, $P = 0.013$) and lymph node positivity at the time of surgery (OR = 5.7, 95% CI 2.0–16.1, $P = 0.001$). CXC chemokine receptor 7 expression was observed more frequently in squamous carcinoma than in adeno-/adenosquamous carcinoma (OR = 2.6, 95% CI 1.1–6.0, $P = 0.025$). Both EGFR and CXCR4 expression were observed more frequently in squamous/adenosquamous carcinoma than in adenocarcinoma

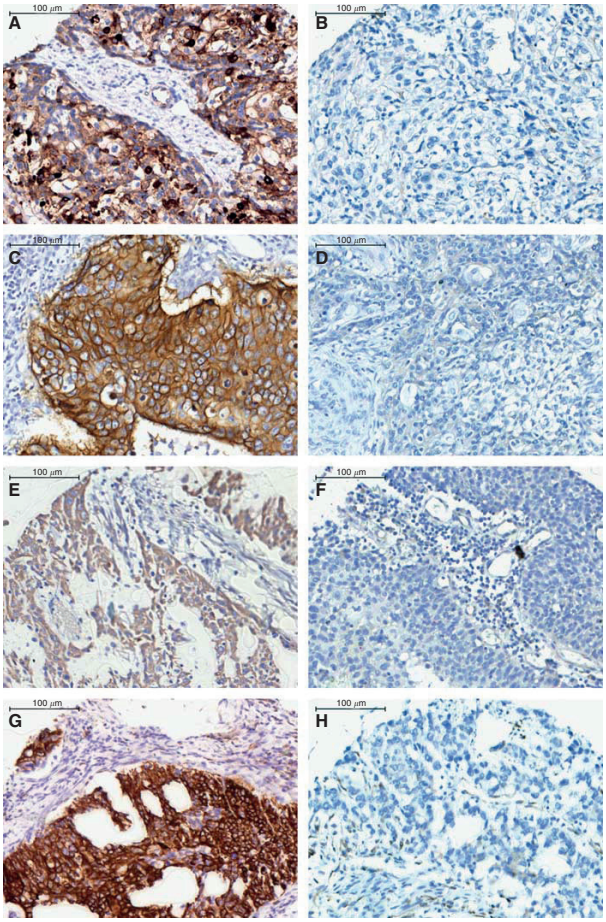

Figure 1 Representative examples of positive (**A**) and negative (**B**) CXCR7 staining, positive (**C**) and negative (**D**) EGFR staining, positive (**E**) and negative (**F**) CXCR4 staining, and positive (**G**) and negative (**H**) CXCL12 staining in the epithelial compartment of squamous cell carcinoma of the cervix.

(OR 26.6, 95% CI 3.3–217.4, $P<0.001$ and OR = 6.4, 95% CI 1.6–25.7, $P=0.008$, respectively). No other associations were observed between CXCR7, CXCR4, CXCL12 and clinicopathological characteristics. As previously described, EGFR expression was associated with tumour size, parametrial invasion, vasoinvasion and lymph node metastasis (Schrevel *et al*, 2011).

Disease-specific and disease-free survival for CXCR7, EGFR, CXCR4 and CXCL12

Median follow-up time was 137 months (range 5–266) for all patients and 156 months (range 8–266) for patients alive at the time of data collection. Of the 24 patients with disease recurrence, a combination of local recurrences ($n=4$), regional recurrences ($n=3$) and distant metastases ($n=20$) was observed. Of the 34 patients who died during the follow-up period, 22 deaths could be attributed to cervical cancer. Five-year disease-free and disease-specific survival rates for the whole group were 77% (s.e. = 4) and 79% (s.e. = 4), respectively. Univariate Cox-regression analysis for all clinicopathological characteristics and disease-free survival is shown in Table 2. Multivariate regression analysis for all clinicopathological characteristics showed that only tumour size (hazard ratio (HR) = 4.8, 95% CI 1.8–12.3, $P=0.001$) and lymph node metastasis (HR = 5.4, 95%

Table 1 CXCR7, EGFR, CXCR4 and CXCL12 expression in cervical cancer patients

Expression	N (%)[a]
CXCR7	
Negative	58 (57)
Weak	18 (18)
Moderate	20 (20)
Strong	5 (5)
EGFR	
Negative	19 (20)
Weak	16 (17)
Moderate	31 (33)
Strong	28 (30)
CXCR4	
Negative	36 (37)
Weak	51 (53)
Moderate	10 (10)
Strong	0 (0)
CXCL12	
Negative	13 (14)
Weak	35 (38)
Moderate	28 (31)
Strong	16 (17)

Abbreviations: CXCR = CXC chemokine receptor; EGFR = epidermal growth factor receptor. [a]Total number of assessed cases is 101 for CXCR7, 94 for EGFR, 97 for CXCR4 and 92 for CXCL12. Protein expression was determined through analysis of an immunohistochemically stained tissue array, as described in the Materials and Methods section. Immunoreactivity was scored as negative, weak, moderate or strong staining intensity.

CI 2.3–12.7, $P<0.001$) were independently associated with disease-free survival.

Univariate Cox-regression analysis for CXCR7 showed a strong association between positive CXCR7 expression and disease recurrence, with 63% *vs* 90% disease-free survival for CXCR7-positive and CXCR7-negative cases, respectively, (HR = 4.3, 95% CI 1.7–11.0, $P=0.002$, Figure 2A, Table 3). CXC chemokine receptor 7 expression was also associated with disease-specific survival (HR = 3.9, 95% CI 1.5–10.2, $P=0.005$). CXC chemokine receptor 4 and CXCL12 expression were not associated with disease-free (Table 3) nor disease-specific survival (data not shown). There was a trend for a positive association between EGFR expression and disease-free survival (HR = 2.3, 95% CI 0.9–6.4, $P=0.095$, Table 3).

Multivariate regression analysis showed that only CXCR7 expression (HR = 3.4, 95% CI 1.1–10.5, $P=0.030$), tumour size per mm increase in tumour size (HR = 1.1, 95% CI 1.0–1.1, $P=0.001$) and lymph node metastasis at the time of surgery (HR = 4.5, 95% CI 1.2–16.8, $P=0.024$) were independent predictors of disease-free survival, when correcting for HPV-type, histopathological diagnosis, tumour size, infiltration depth, parametrial invasion, vasoinvasion, lymph node metastasis, resection margins and postoperative radiotherapy. Multivariate regression analysis for CXCR7, EGFR, CXCR4 and CXCL12 expression showed that only CXCR7 was independently associated with disease-recurrence (HR = 4.2, 95% CI 1.5–12.0, $P=0.007$).

Disease-free survival for co-expression of CXCR7, EGFR, CXCR4 and CXCL12

To assess whether co-expression of CXCR7 and its ligand CXCL12 was associated with disease-free or disease-specific survival, groups were made for CXCR7-negative cases ($n=58$) and CXCR7-positive cases with ($n=33$) or without CXCL12 expression

Table 2 Disease-free survival for clinicopathological parameters

Variables	N[a]	DFS (%)	HR	95% CI	P-value
HPV-type					
Negative	9	89	—	—	—
16	54	76	2.2	0.3–16.3	0.465
18	23	65	3.7	0.5–29.7	0.216
Other	17	88	1.1	0.1–12.0	0.944
Histopathology					
SCC	62	74	0.7	0.3–1.7	0.447
A(S)	41	81			
Tumour size					
<40 mm	59	90	6.1	2.4–15.6	**<0.001**
$\geqslant 40$ mm	38	55			
Infiltration depth					
<15 mm	56	88	3.7	1.5–9.0	**0.003**
$\geqslant 15$ mm	46	63			
Parametrial invasion					
Negative	94	81	5.2	2.0–13.1	**0.001**
Positive	9	33			
Vasoinvasion					
Negative	48	83	2.1	0.9–4.9	0.086
Positive	52	69			
Lymph node metastasis					
Negative	79	87	7.2	3.2–16.3	**<0.001**
Positive	24	42			
Resections margins					
Negative	77	83	3.0	1.4–6.8	**0.007**
Positive	26	58			
Postoperative radiotherapy					
No	47	92	5.1	1.7–15.0	**0.003**
Yes	56	64			

Abbreviations: A(S) = adeno(squamous) carcinoma; DFS = disease-free survival; HR = hazard ratio; 95% CI = 95% confidence interval; HPV = human papillomavirus; SCC = squamous cell carcinoma. Univariate Cox-regression analysis for disease-free survival based on clinicopathological parameters. [a]Total number of cases = 103; for some variables, data were not available for all patients. The bold entries place emphasis on statistically significant *P*-values.

($n=5$). Survival analysis showed no difference between CXCR7-positive/CXCL12-positive and CXCR7-positive/CXCL12-negative cases (data not shown). To investigate whether co-expression of CXCR7 and EGFR, CXCR4 or both was associated with disease-free or disease-specific survival, groups were made for CXCR7-negative cases ($n=58$), CXCR7-positive cases with no EGFR or CXCR4 expression (single positive, $n=4$), CXCR7-positive cases with either EGFR or CXCR4 expression (double positive, $n=16$) and CXCR7-positive cases with both EGFR and CXCR4 expression (triple positive, $n=21$). As the survival curves for the 4 CXCR7 single-positive cases were comparable to the 58 CXCR7-negative cases, these groups were combined for further analyses. Univariate Cox-regression analysis showed equally increased risks of disease recurrence for both double- and triple-positive cases when compared with CXCR7-negative/single-positive cases (HR = 3.6, 95% CI 1.2–10.7, $P=0.022$, and HR = 4.2, 95% CI 1.5–11.7, $P=0.006$, respectively, Figure 2B, Table 3). As expression of CXCR7 was observed more frequently in squamous cell carcinoma than in other histopathological types, regression analysis was also performed for this subgroup. In squamous cell carcinomas, the risk between disease-free survival and CXCR7-negative/single-positive cases, double-positive cases and triple-positive cases increased in an additive fashion (double: HR = 2.7, 95% CI 0.6–12.1, $P=0.193$, triple: HR = 5.8, 95% CI 1.5–22.4, $P=0.011$, Figure 2C, Table 3). Survival analysis for disease-specific survival showed similar results (data not shown).

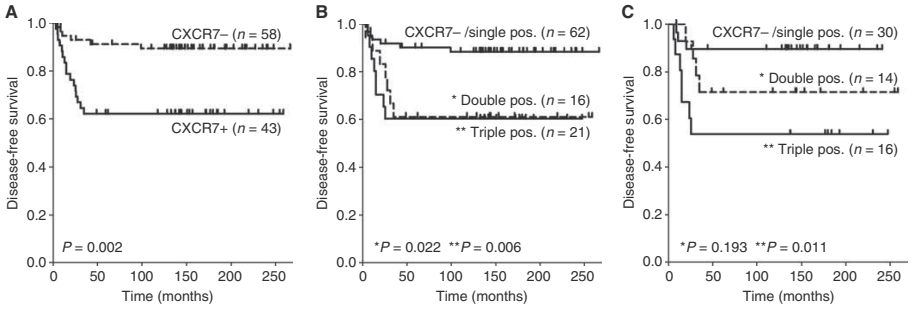

Figure 2 Disease-free survival in cervical cancer patients with positive or negative CXCR7 expression (**A**), CXCR7-negative/CXCR7 single-positive cases, CXCR7–positive, and either EGFR- or CXCR4-positive (double positive) cases and CXCR7-, EGFR- and CXCR4-positive (triple positive) cases in all patients (**B**) and in patients with squamous cell carcinoma (**C**). *P*-values were obtained using Cox-regression analysis; see Table 3 for HRs and CIs.

Table 3 Disease-free survival for CXCR7, EGFR, CXCR4 and CXCL12 protein expression

Variables	*N* [a]	DFS (%)	HR	95% CI	*P*-value
CXCR7					
Negative	58	90	4.3	1.7–11.0	**0.002**
Positive	43	63			
EGFR					
Low	35	86	2.3	0.9–6.4	0.095
High	59	71			
CXCR4					
Negative	36	78	1.2	0.5–2.9	0.625
Positive	61	74			
CXCL12					
Negative	13	69	0.8	0.3–2.3	0.653
Positive	79	75			
CXCR7/EGFR/CXCR4					
CXCR7 negative/single positive	62	89	—	—	—
Double positive	16	63	3.6	1.2–10.7	**0.022**
Triple positive	21	62	4.2	1.5–11.7	**0.006**
CXCR7/EGFR/CXCR4 in SCC					
CXCR7 negative/single positive	30	90	—	—	—
Double positive	14	71	2.7	0.6–12.1	0.193
Triple positive	16	56	5.8	1.5–22.4	**0.011**

Abbreviations: CXCR = CXC chemokine receptor; DFS = disease-free survival; EGFR = epidermal growth factor receptor; HR = hazard ratio; 95% CI = 95% confidence interval; SCC = squamous cell carcinoma. [a]Total number of cases = 103; for some variables, data were not available for all patients. Univariate Cox-regression analysis for disease-free survival based on the status of CXCR7, EGFR, CXCR4 and/or CXCL12 protein expression. CXCR7, CXCR4 and CXCL12 expression are divided into negative (intensity score of 0) and positive (intensity scores of 1, 2 and 3) groups. EGFR expression was divided into low (intensity scores of 0 and 1) and high (intensity scores of 2 and 3) expression groups. In addition, co-expression of CXCR7, EGFR and CXCR4 was analysed as follows: CXCR7-negative cases ($n = 58$) and CXCR7 single-positive cases ($n = 4$) were combined and compared with double-positive cases (i.e. CXCR7-positive cases with either high EGFR expression or positive CXCR4 expression), and triple positive cases (i.e. CXCR7-positive cases with both high EGFR expression and positive CXCR4 expression). This analysis was also performed after selection of patients with SCC. The bold entries place emphasis on statistically significant *P*-values.

DISCUSSION

The present study showed for the first time that CXCR7 was independently associated with disease recurrence in cervical cancer. In addition, CXCR7 expression was associated with tumour size and lymph node status at the time of surgery. These findings suggest that CXCR7 expression leads to more aggressive tumour growth and metastasis formation.

CXC chemokine receptor 7 is frequently expressed on embryonic and neoplastic transformed cells, but undetectable or expressed at very low levels in normal adult tissues (Burns *et al*, 2006; Miao *et al*, 2007). Experiments using mouse models showed that increased CXCR7 expression resulted in a larger tumour size and an enhanced metastatic potential (Miao *et al*, 2007). Previously, CXCR7 has been shown to be associated with disease-specific and disease-free survival in non-small cell lung cancer (Iwakiri *et al*, 2009). In the present study, we showed that in cervical cancer as well, disease-free survival was lower in CXCR7-positive cases (63%) than in CXCR7-negative cases (90%, HR = 4.3, 95% CI 1.7–11.0, $P = 0.002$), even when adjusting for other prognostic factors. Disease-specific survival was also strongly associated with CXCR7 expression. Therefore, CXCR7 might be used as a prognostic marker to predict disease recurrence, with additive predictive value to lymph node status and tumour size.

The current data also showed that CXCR7 was often co-expressed with EGFR or CXCR4 (double positive) or both (triple positive), with only four single-positive cases, supporting the hypothesis that CXCR7 requires other molecules for optimal intracellular signalling. Sierro *et al* (2007) have shown that CXCR7 forms heterodimers with CXCR4 and Singh and Lokeshwar (2011) have shown that CXCR7 is capable of forming heterodimers with EGFR. Activation of HEK293 cells co-expressing CXCR7 and CXCR4 results in a stronger calcium flux when compared with cells expressing CXCR4 alone, whereas CXCR7 was unable to induce calcium flux by itself (Burns *et al*, 2006; Sierro *et al*, 2007). However, CXCR7 is capable of signalling through MAPK/AKT pathways, indicating that EGFR, which also signals through MAPK/AKT, might be an alternative co-receptor for CXCR7 (Grymula *et al*, 2010). Interestingly, in CXCR7- and CXCR4-positive rhabdomyosarcoma cells, CXCL12-induced chemotaxis could still be observed after blocking CXCR4, although strongly reduced when compared with controls (Grymula *et al*, 2010). Similarly, in CXCR7-positive, CXCR4-negative 4T1 breast cancer cells, CXCR7 expression promoted tumour growth and progression of lung metastases in mice (Miao *et al*, 2007). As rhabdomyosarcoma cells and 4T1 breast cancer cells are both EGFR-positive, CXCR7 might signal through heterodimerisation with EGFR in case CXCR4 is absent or blocked (Dykxhoorn *et al*, 2009; Herrmann *et al*, 2010). However, although our data support this hypothesis, as a positive correlation was observed between CXCR7 and EGFR expression, functional studies on cervical cancer

cell lines are required to determine whether CXCR7 and EGFR are indeed co-dependent for signal transduction. Furthermore, although HRs were similar for double- and triple-positive cases in the whole study group, HRs increased in an additive fashion in squamous cell carcinomas, which indicates that molecular mechanisms regulating metastasis formation may differ between histopathological subtypes.

In conclusion, CXCR7 expression was strongly associated with tumour size, lymph node metastasis, disease recurrence and poor disease-specific survival, suggesting that CXCR7 expression leads to a biologically more aggressive tumour. CXC chemokine receptor 7 might be a promising new prognostic marker in cervical cancer and may serve as a potential therapeutic target. CXC chemokine receptor 7 is often co-expressed with CXCR4 and/or EGFR, which supports the hypothesis that these receptors assist in CXCR7 signal transduction.

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Chapter 9

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[1]. 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[2]. 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 [3-5]. 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[6, 7]. 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.

1 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[8] is consistent with work by others showing that *TLR4* was only found in HaCat cells, but not in primary human KCs[9]. 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[10-16] 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.,[12] because one of the normal KC lines (NHK1) is *TLR9* positive, while the second line (NHK2) is *TLR9* negative.

2 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[17, 18] 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[19]. 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,

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[10, 12, 13, 20]. 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. [10] 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[12]. 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.[12, 13] and as

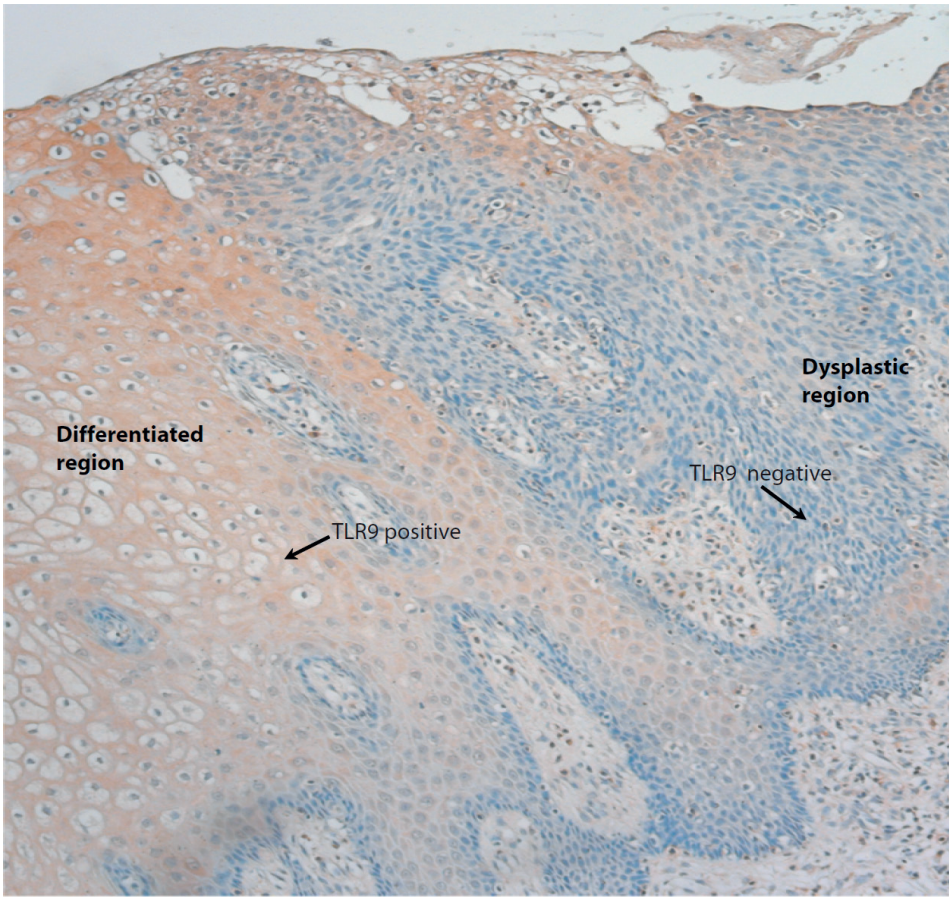

Figure 1 | TLR9 expression in cervical intraepithelial neoplasia 3 (CIN3)

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.[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[12], Lee et al. reported that such samples show greatly increased TLR9 staining[20]. A peculiar feature of the data by Hasan et al.[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[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[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 *BAMBI*, a negative regulator of TGF-β. The well known immuosuppressive roles of TGF-β 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[22], MIP3A[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 *IL1B and 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 [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[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.[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[27] analyzed our genome-wide data using approaches like KEGG database for pathway analyses, CORE_TF[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[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[30] 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[31]. Our gene lists in enriched protein ubiqutination 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[25], 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[32]. 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[32]. 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[32]. It is known that hrHPVs inhibit antigen presentation to cytotoxic cells[33], 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 keratinocyes 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-

CSF to the culture[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[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 keratinocyes 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)[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[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[39] suggesting that UCHL1 expression is lost during cervical tumorigenesis. Chronic inflammation has been linked to the development of cancer[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[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

shows that HPV E6 mediates proteasomal degradation of CYLD in cervical cancer cell lines, thereby allowing hypoxia-induced NF-κβ activation and tumorigenesis[41]. Since UCHL1 expression is undetectable in almost all cervical cancer cell lines[39], 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 opportunist 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[42]. 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.,[42] 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 [43, 44]. Interestingly, hrHPV-specific T cells infiltrating cervical cancer and lymph nodes are predominantly restricted via HLA-DQ and –DP [45], 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 IL1B, 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 wells 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)[46]. Similar approaches are also successfully tested to treat the HPV-infected region of cervix [47]. 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[48]. 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 [49] are currently known that interfere with the activity of UCHL1. Anti-sense oligonuleotides 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[50] 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 [51], 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.,[52] 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.,[53]. 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,[54], 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[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[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 [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[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[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[60] [61] to conclusively phenotype the nature of PD-1-postive 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 [62]. 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 [7]. 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 [63]. 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[6], 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 [61]. 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[58] 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.

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Supporting Information

Supporting Information Chapter 2

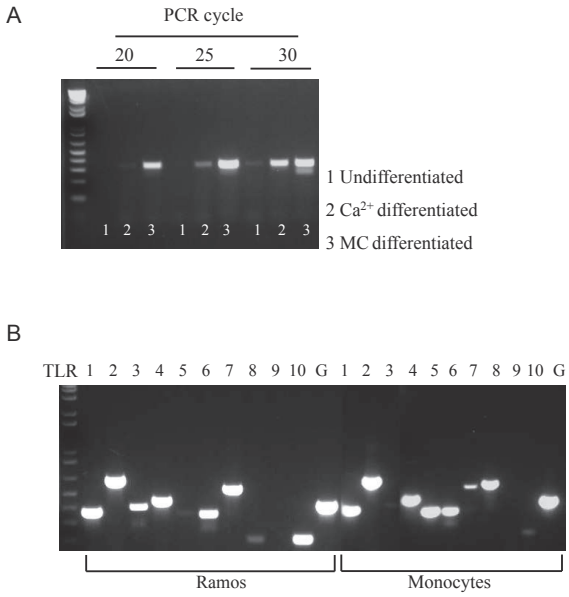

Figure S1 | Positive controls for keratinocyte differentiation and PRR expression. (A), Reverse transcription PCR detection of the small proline-rich protein 2A (SPRR2A), a molecular marker of KC differentiation after 20, 25 and 30 PCR cycles in undifferentiated (1), partially differentiated (2) and fully differentiated (3) normal foreskin keratinocytes. SPRR2A expression was absent from undifferentiated KCs, low in Ca2+-treated KCs and high in KCs cultured in suspension with Ca2+ and methylcellulose, confirming that the KCs consisted of undifferentiated (basal) cells and differentiated in vitro. (B), Reverse transcription PCR detection of TLRs 1-10 and GAPDH ("G") in mRNA samples from Ramos B-cells and monocytes.

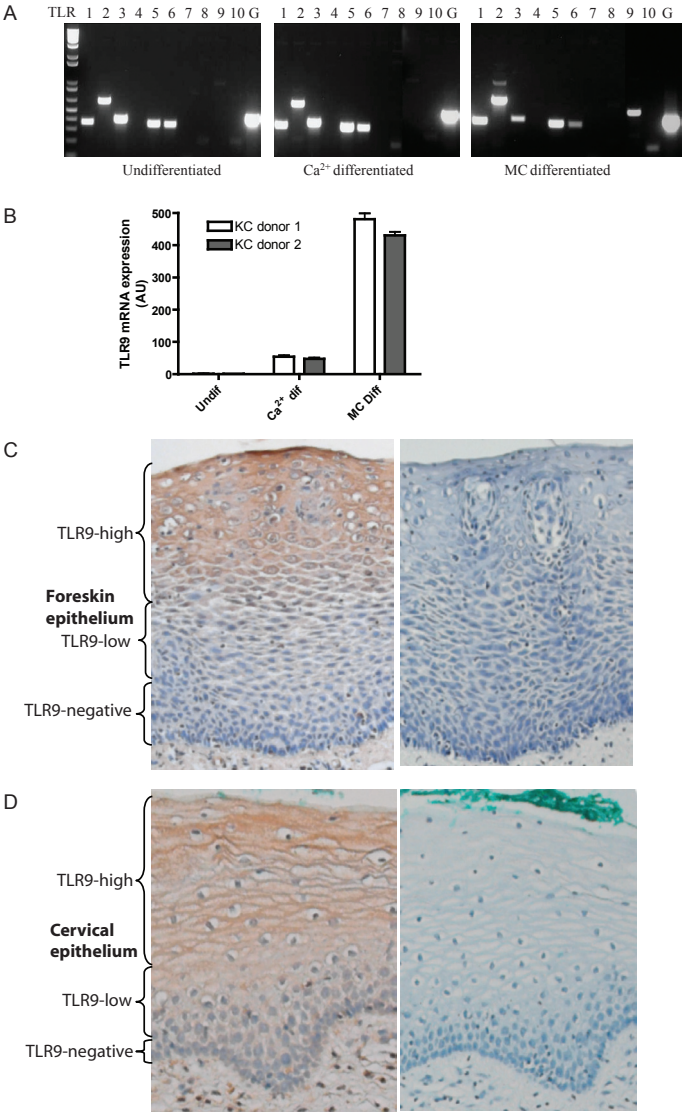

Figure S2 | TLR9 expression in stratified squamous epithelia progressively increases with KC differentiation stage. (A), Total RNA of the indicated cells was subjected to RT-PCR (35 cycles) with specific primers human TLR1-10 or GAPDH as indicated by a "G". (B), TaqMan real-time PCR was performed for TLR9 on total RNA samples from indicated cell types. TLR9 expression was normalized against GAPDH mRNA levels. Data represent an average of three independent experiments. (C), Immunohistochemical staining of paraffin-embedded healthy foreskin sections and (D) sections of healthy ectocervical epithelium with human TLR9-specific monoclonal antibody (left panels) or isotype control antibody (right panels) in combination with peroxidase-conjugated secondary antibody. Cell nuclei were counterstained with haematoxylin. Original magnification 125X. Stainings shown are representative of at least three samples of different origin.

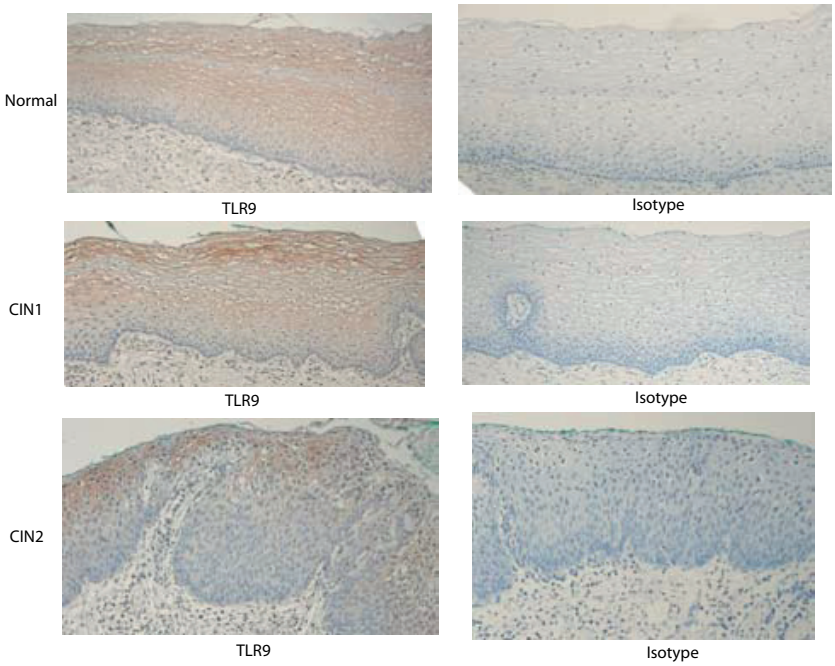

Figure S3 | TLR9 is expressed in differentiated cell layers of HPV-positive cervical epithelial neoplasia. Immunohistochemical staining with TLR9-specific or isotype control antibody of paraffin-embedded sections of normal and dysplastic genital epithelia. Staining was performed as described in the legend to Figure S2. Original magnification 125X. Sections of the following epithelial samples are shown: A) normal cervical epithelium, B) CIN1, C) CIN2.

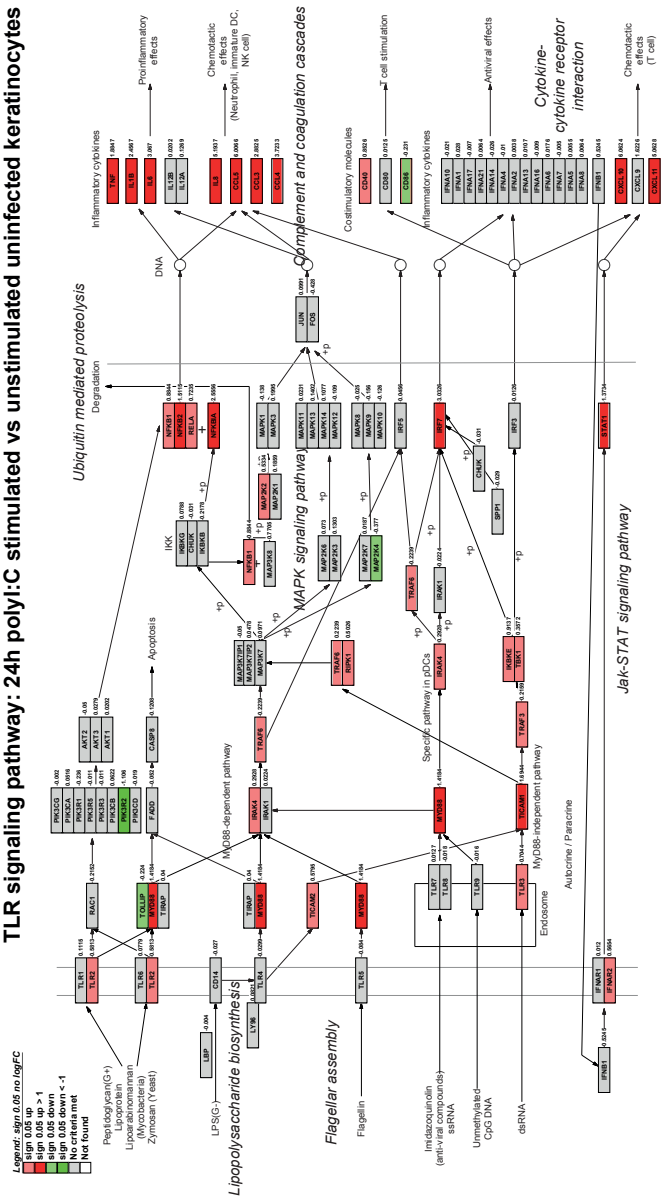

Figure S4 | TLR signalling in KCs. Toll-like receptor signalling pathway (KEGG hsa4620) overlaid with differentially expressed genes between 24 hrs poly(I:C) stimulated and unstimulated uninfected keratinocyte cultures. Differentially expressed genes (FDR ≤ 0.05) were colored bright red (log2 fold change ≥ 1) or dim red (log2 fold change between 0 and 1) for upregulation upon poly(I:C) stimulation, or bright green (log2 fold change ≤ -1) or dim green (log2 fold change between 0 and -1) for downregulation. Grey boxes represent genes not fulfilling the above criteria, while white boxes are genes not represented by probes on the array.

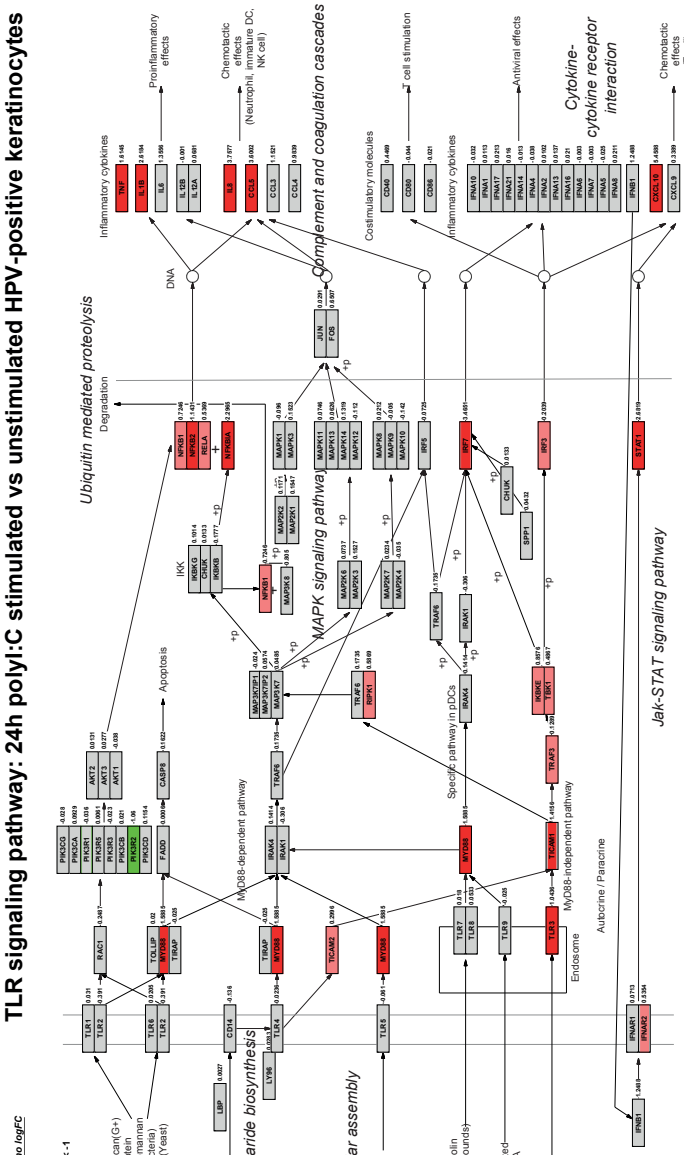

Figure S5 | TLR signalling in HPV-KCs. Toll-like receptor signalling pathway (KEGG hsa4620) overlaid with differentially expressed genes between 24 hrs poly(I:C) stimulated and unstimulated HPV-infected keratinocyte cultures. For explanation of colors, see Figure S4.

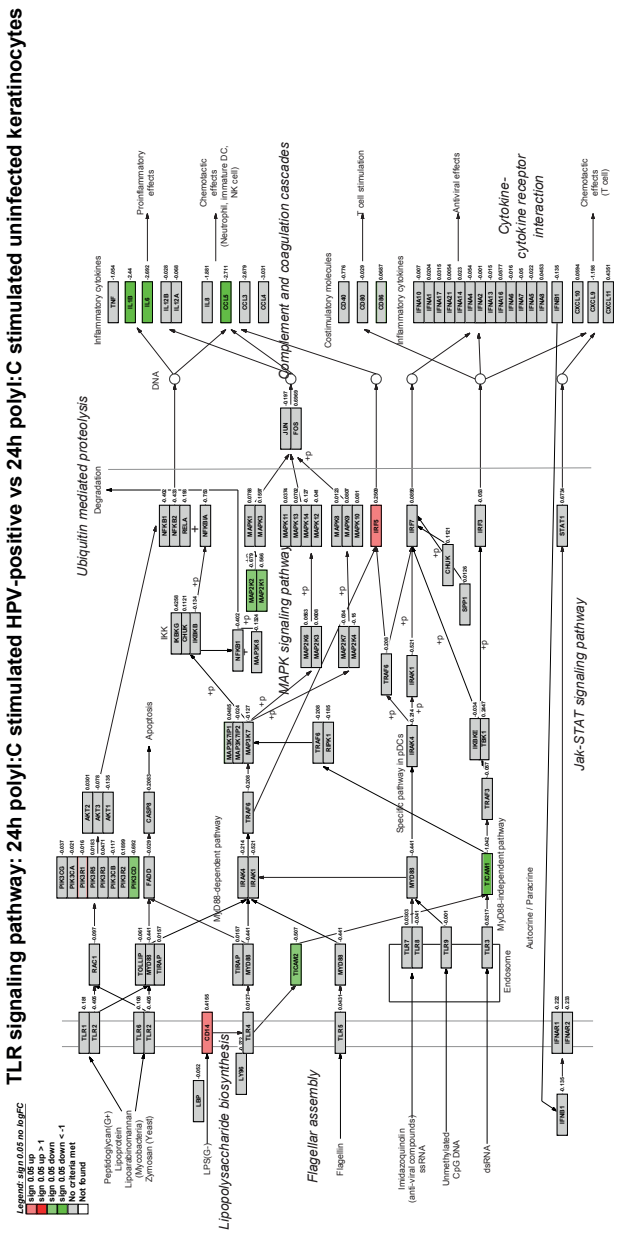

Figure S6 | Differential TLR signalling between HPV-KCs and KCs. Toll-like receptor signalling pathway (KEGG hsa4620) overlaid with differentially expressed genes between HPV-infected and uninfected keratinocytes, both after 24 hrs poly(I:C) stimulation. Differentially expressed genes (FDR ≤ 0.05) were colored according to their log2 fold change (see legend Figure S4) for upregulation (red) or downregulation (green) in HPV-positive cells.

Table S1 | Differential expression of pattern recognition receptors and signalling molecules in HPV-infected and uninfected keratinocytes.

		HPV-infected versus Uninfected*				Polyl:C Stimulation**		
Gene Symbol	**ProbeID**	**Unstimulated**	**4 hrs Polyl:C**	**24 hrs Polyl:C**	**Change*** **	**Uninfected**	**HPV-infected**	**Change**
TLRs								
TLR1	ILMN_1731048	0.5075	0.8101	0.1245		0.0639	0.9252	
TLR2	ILMN_1772387	0.6670	0.7159	0.2514		**0.0116**	0.1590	**up**
TLR3	ILMN_1689578	0.8196	0.9781	0.2745		**0.0179**	**0.0075**	**up**
TLR4	ILMN_1706217	0.8120	0.8130	0.8617		0.4416	0.6560	
TLR5	ILMN_1722981	0.9297	0.9036	0.7692		0.3101	0.5266	
TLR6	ILMN_1749287	0.8026	0.9457	0.4102		0.2668	0.9707	
TLR7	ILMN_1677827	0.7841	0.7538	0.6670		0.1113	0.8872	
TLR8	ILMN_1682251	0.5907	0.6089	0.5755		0.4787	0.1055	
TLR8	ILMN_1657892	0.9512	0.6378	0.6467		0.8404	0.4786	
TLR8	ILMN_1705047	0.9912	0.9477	0.9704		0.8519	0.7386	
TLR9	ILMN_1679798	0.9517	0.9791	0.9929		0.8768	0.7921	
TLR10	ILMN_1719905	0.2354	0.7753	0.5113		0.6290	0.9121	
Virus PRRs								
DDX58/RIG-I	ILMN_1797001	0.9137	0.8457	0.5615		**0.0002**	**0.0004**	**up**
IFIH1/MDA5	ILMN_1781373	0.8513	0.9743	0.9656		**0.0001**	**0.0001**	**up**
EIF2AK2/PKR	ILMN_1706502	0.3856	0.4664	0.8941		0.0970	**0.0128**	**up**
NLRP3	ILMN_1712026	0.1002	**0.0261**	**0.0199**	down	0.0620	0.4713	
NLRP3	ILMN_1713379	0.8305	0.1922	0.5545		0.5681	0.6947	
Adaptors								
MYD88	ILMN_1738523	0.4745	0.4618	0.6085		**0.0061**	**0.0071**	**up**
TICAM1/TRIF	ILMN_1724863	0.0865	0.2568	**0.0178**	**down**	**0.0001**	**0.0006**	**up**
TICAM1/TRIF	ILMN_1815079	0.8277	0.6189	0.4977		0.0595	0.2895	
TICAM2/TRAM	ILMN_1651346	0.3780	0.3253	**0.0209**	down	**0.0007**	0.0664	up
IRFs								
IRF1	ILMN_1708375	0.5398	0.7765	0.9370		**0.0000**	**0.0002**	**up**
IRF2	ILMN_1765547	0.9592	0.5474	0.8788		0.1001	0.8366	
IRF3	ILMN_1765649	0.0521	0.3810	0.7662		0.8172	**0.0332**	up
IRF4	ILMN_1754507	0.5804	0.9340	0.9360		0.9231	0.6536	
IRF5	ILMN_1670576	**0.0358**	**0.0145**	**0.0179**	up	0.7228	0.5558	
IRF6	ILMN_1725946	0.0830	0.1206	0.0761		**0.0023**	**0.0053**	**up**
IRF7	ILMN_1674646	0.4028	0.2741	0.3775		**0.0001**	**0.0001**	**up**
IRF7	ILMN_1798181	0.9638	0.9692	0.7348		**0.0140**	**0.0002**	**up**
IRF8	ILMN_1666594	0.9293	0.9970	0.8020		0.8348	0.8932	

* P-values were calculated using a linear model (Smyth, 2004) and adjusted for multiple testing according to Benjamini and Hochberg (1995). P-values below 0.05 are in bold.

** The 4 hrs versus unstimulated and 24 hrs versus unstimulated comparisons were combined into one F-test using limma.

*** Change indicates direction of expression change. Changes that exceed the arbitrary biological significance threshold of logFC 1are in bold.

Table S2 | HPV signature genes. (modified table to obtain 25 most downregulated and 25 most upregulated genes respectively)

Log2 fold changes from limma contrasts, all compared to Uninfected unstimulated						Probe annotation
Uninf unstim	Uninf 4 hrs polyI:C	Uninf 24 hrs polyI:C	HPV-inf unstim	HPV-inf 4 hrs polyI:C	HPV-inf 24 hrs polyI:C	ILMN_GENE
0	0,02	-0,91	-5,02	-5,16	-5,30	VIM
0	-0,07	-0,73	-3,44	-3,51	-3,57	C7ORF10
0	-0,07	-0,35	-1,79	-1,81	-1,84	NDN
0	-0,13	-0,55	-2,16	-2,17	-2,11	NDRG1
0	-0,08	-1,16	-4,42	-4,38	-4,80	SERPINE2
0	-0,08	-0,41	-1,45	-1,51	-1,54	HS3ST2
0	-0,02	-0,33	-1,10	-1,20	-1,29	LOC387882
0	0,06	-0,28	-1,20	-1,13	-1,20	SEPP1
0	0,16	-0,17	-0,97	-0,93	-1,04	FEZ1
0	0,27	-0,20	-3,00	-2,89	-3,26	COL8A1
0	0,05	-0,39	-2,87	-2,96	-3,10	SRPX
0	-0,02	-0,18	-1,91	-1,95	-1,95	NLRP2
0	-0,12	-0,29	-2,12	-2,30	-2,73	FTL
0	-0,15	-0,38	-2,55	-2,60	-2,96	GPNMB
0	0,00	-0,13	-1,43	-1,43	-1,62	COL5A1
0	-0,01	-0,09	-0,93	-0,99	-1,09	KHDRBS3
0	-0,31	-0,57	-1,95	-2,04	-2,56	PRAC
0	-0,31	-0,84	-2,73	-2,98	-3,20	DPYSL2
0	-0,20	-0,52	-1,49	-1,55	-1,64	ERCC1
0	-0,27	-0,57	-1,58	-1,83	-1,91	DCBLD2
0	-0,20	-0,49	-1,17	-1,23	-1,42	LEPREL2
0	-0,39	-0,36	-1,56	-2,33	-2,12	NUAK1
0	-0,08	-0,12	-1,10	-1,44	-1,10	LPIN1
0	-0,33	-0,34	-0,99	-1,24	-1,50	TACC1
0	-0,20	-0,28	-0,81	-0,95	-1,36	CALU
0	-0,37	-0,13	-0,81	-1,08	-1,36	E2F7
0	0,02	0,07	0,48	0,56	1,55	DUOXA2
0	-0,37	0,79	1,34	0,21	0,97	FOXQ1
0	-0,04	1,05	0,76	0,56	2,07	FBXO32
0	0,01	1,56	1,27	1,41	1,91	ASS1
0	-0,19	2,50	2,19	2,21	2,87	CLDN7
0	0,08	2,17	1,38	1,68	2,96	ELF3
0	0,24	2,37	1,81	1,90	3,00	RHCG
0	-0,04	1,00	1,43	1,43	2,31	GPRC5A
0	-0,01	1,50	1,71	1,82	2,99	S100A9
0	-0,26	1,23	1,39	1,72	3,16	S100A8
0	-0,23	0,90	1,60	1,53	2,84	DHRS9
0	0,03	0,92	1,65	1,74	2,73	MYEOV
0	-0,05	1,78	3,12	3,02	4,39	MDK
0	-0,26	1,96	3,28	3,31	4,96	LCN2
0	0,96	1,36	0,54	1,38	2,88	TRIM5
0	0,74	2,24	0,99	1,90	2,44	CXCL16
0	0,54	1,29	1,23	2,05	3,39	FLJ20035
0	0,37	1,48	1,52	2,20	3,70	SLC6A14
0	1,48	2,27	2,20	3,13	4,39	RARRES3
0	1,46	2,54	2,31	3,57	3,79	HES4
0	2,11	0,85	0,97	3,16	2,31	SDC4
0	2,35	0,92	1,04	3,10	2,70	ZC3HAV1
0	2,57	0,91	1,07	3,31	2,78	ZC3HAV1
0	2,30	1,87	0,97	4,16	2,91	TNFAIP2
0	5,74	4,67	1,56	6,00	5,51	IFIT2

Table S3 | Enrichment of transcription factor binding sites in HPV signature gene promoters.

A. Expression Clusters 1-3, downregulated in HPV-positive keratinocytes

Expression Cluster 1 (115 promoters)				Expression Cluster 2 (153 promoters)				Expression Cluster 3 (12 promoters)			
matrix*	# exp**	freq exp	freq ran***	matrix	# exp	freq exp	freq ran	matrix	# exp	freq exp	freq ran
AHR_Q5	6	0.0522	0.0182	***AP1_Q2_01***	22	0.1438	0.0819	AP2ALPHA_01	5	0.4167	0.1618
AP1_Q2_01	17	0.1478	0.0819	**AP1_Q4_01**	12	0.0784	0.0293	**CEBP_Q2_01**	7	0.5833	0.2357
AP1_Q6	21	0.1826	0.1062	***AP1_Q6***	26	0.1699	0.1062	**CREL_01**	3	0.2500	0.0738
AP2_Q3	74	0.6435	0.4440	**AP1_Q6_01**	20	0.1307	0.0681	**DBP_Q6**	5	0.4167	0.1581
AP2_Q6	60	0.5217	0.3439	AP2_Q3	87	0.5686	0.4440	**E2_Q6_01**	1	0.0833	0.0051
AP2ALPHA_02	43	0.3739	0.2569	AP2_Q6_01	74	0.4837	0.3560	**EGR3_01**	7	0.5833	0.2610
AP2GAMMA_01	46	0.4000	0.3007	***CACD_01***	89	0.5817	0.4872	**GFI1B_01**	3	0.2500	0.0738
BACH2_01	6	0.0522	0.0061	***EGR_Q6***	23	0.1503	0.0860	**HP1SITEFACTOR_Q6**	1	0.0833	0.0115
CACD_01	71	0.6174	0.4872	GC_01	77	0.5033	0.3941	HSF2_01	2	0.1667	0.0260
CBF_01	26	0.2261	0.1396	**HIC1_03**	33	0.2157	0.1463	**IK1_01**	5	0.4167	0.1642
DEC_Q1	8	0.0696	0.0307	HSF1_Q6	1	0.0065	0.0003	**NFKAPPAB65_01**	2	0.1667	0.0266
EGR_Q6	18	0.1565	0.0860	KROX_Q6	41	0.2680	0.1800	**PITX2_Q2**	2	0.1667	0.0243
EGR1_01	31	0.2696	0.1790	**NFY_01**	16	0.1046	0.0583	**RREB1_01**	1	0.0833	0.0040
ETF_Q6	61	0.5304	0.3982	**NGFIC_01**	46	0.3007	0.2023	**SMAD_Q6**	6	0.5000	0.2073
ETS_Q6	28	0.2435	0.1571	SP1_Q2_01	55	0.3595	0.2687	**SREBP1_02**	8	0.6667	0.3645
ETS1_B	42	0.3652	0.2653	SP1_Q4_01	62	0.4052	0.3186	**STAT5A_01**	2	0.1667	0.0361
EVI1_01	3	0.0261	0.0071	SP1_Q6	61	0.3987	0.3007				
GC_01	75	0.6522	0.3941	***SP1_Q6_01***	68	0.4444	0.3109				
HEN1_01	1	0.0087	0.0003	**ZNF219_01**	6	0.0392	0.0152				
HIC1_02	24	0.2087	0.1335								
HMX1_01	2	0.0174	0.0013								
HNF4_01	9	0.0783	0.0354								
KROX_Q6	37	0.3217	0.1800								
LRF_Q2	45	0.3913	0.2471								
MAZ_Q6	49	0.4261	0.2980								
MUSCLE_INI_B	47	0.4087	0.2933								
MZF1_02	45	0.3913	0.2873								
NRSF_01	8	0.0696	0.0310								
PAX5_01	73	0.6348	0.4673								
POLY_C	1	0.0087	0.0003								
SP1_Q2_01	48	0.4174	0.2687								
SP1_Q4_01	64	0.5565	0.3186								
SP1_Q6	61	0.5304	0.3007								
SP1_Q6_01	63	0.5478	0.3109								
SP3_Q3	40	0.3478	0.2421								
SREBP1_Q6	43	0.3739	0.2751								
WT1_Q6	34	0.2957	0.1831								

GC_01

*TRANSFAC position weight matrices with a CORE_TF p-value for over-representation ≤ 0.01 and frequency in the random set < 50%.
Matrices unique to one expression cluster are in bold. Matrices shared between two expression clusters (either belonging to 1-3 or 4-6) are in bold and italics.
** Number of expression cluster promoters with a hit for the matrix.
*** Frequency of promoters with a hit for the matrix in 2966 random promoters

B. Expression Clusters 4-6, upregulated in HPV-positive keratinocytes

Expression Cluster 4 (129 promoters)			
matrix	# exp	freq exp	freq ran
ARNT_02	46	0.3566	0.2498
CETS168_Q6	44	0.3411	0.2485
CETS1P54_03	80	0.6202	0.4673
E2F_Q3	18	0.1395	0.0708
E2F_Q3_01	14	0.1085	0.0465
E2F_Q4	37	0.2868	0.1628
E2F1_Q3_01	20	0.1550	0.0877
E2F1_Q6_01	9	0.0698	0.0293
ELK1_02	65	0.5039	0.3871
ETF_Q6	67	0.5194	0.3982
GC_01	68	0.5271	0.3941
HSF2_01	10	0.0775	0.0260
KROX_Q6	34	0.2636	0.1800
MEF2_02	10	0.0775	0.0371
MEF2_04	20	0.1550	0.0762
MUSCLE_INI_B	53	0.4109	0.2933
MYC_Q2	38	0.2946	0.1837
SP1_Q2_01	47	0.3643	0.2687
SP1_Q4_01	56	0.4341	0.3186
SP1_Q6	51	0.3953	0.3007
USF_C	29	0.2248	0.1504
USF2_Q6	45	0.3488	0.2229
VMYB_02	79	0.6124	0.4720

Expression Cluster 5 (85 promoters)			
matrix	# exp	freq exp	freq ran
AP1FJ_Q2	14	0.1647	0.0850
AP2_Q3	51	0.6000	0.4440
AP2_Q6	41	0.4824	0.3439
AP2_Q6_01	44	0.5176	0.3560
AP2ALPHA_01	22	0.2588	0.1618
AR_02	11	0.1294	0.0654
AREB6_03	17	0.2000	0.1035
ARNT_02	31	0.3647	0.2498
CP2_02	35	0.4118	0.2620
ETF_Q6	46	0.5412	0.3982
ETS_Q4	21	0.2471	0.1558
GATA1_01	32	0.3765	0.2633
GC_01	52	0.6118	0.3941
GCM_Q2	7	0.0824	0.0320
HSF1_Q6	1	0.0118	0.0003
LRF_Q2	36	0.4235	0.2471
LUN1_01	3	0.0353	0.0098
MAZ_Q6	42	0.4941	0.2980
MEF3_B	1	0.0118	0.0017
MOVOB_01	43	0.5059	0.3530
MYC_Q2	29	0.3412	0.1837
MYCMAX_01	6	0.0706	0.0243
MZF1_02	36	0.4235	0.2873
NERF_Q2	40	0.4706	0.3479
PAX5_01	53	0.6235	0.4673
POLY_C	1	0.0118	0.0003
PR_01	1	0.0118	0.0010
SP1_Q2_01	33	0.3882	0.2687
SP1_Q4_01	37	0.4353	0.3186
SP1_Q6	38	0.4471	0.3007
SP3_Q3	31	0.3647	0.2421
USF_C	25	0.2941	0.1504
USF_Q6_01	14	0.1647	0.0907
USF2_Q6	31	0.3647	0.2229

Expression Cluster 6 (8 promoters)			
matrix	# exp	freq exp	freq ran
CHX10_01	1	0.125	0.0027
CP2_02	6	0.75	0.2620
CREB_01	5	0.625	0.2424
CREB_Q2	5	0.625	0.2205
CREB_Q4	5	0.625	0.2495
CREB_Q4_01	3	0.375	0.1150
CREBP1_Q2	5	0.625	0.2441
CREBP1CJUN_01	4	0.5	0.1625
EVI1_06	1	0.125	0.0105
ICSBP_Q6	2	0.25	0.0212
IRF_Q6	2	0.25	0.0169
IRF2_01	2	0.25	0.0270
IRF7_01	4	0.5	0.1015
ISRE_01	1	0.125	0.0078
KAISO_01	1	0.125	0.0172
LRF_Q2	5	0.625	0.2471
MAF_Q6_01	4	0.5	0.1962
SRF_C	1	0.125	0.0047
SRF_Q5_01	1	0.125	0.0088
SRF_Q6	1	0.125	0.0105

Supporting Information Chapter 3

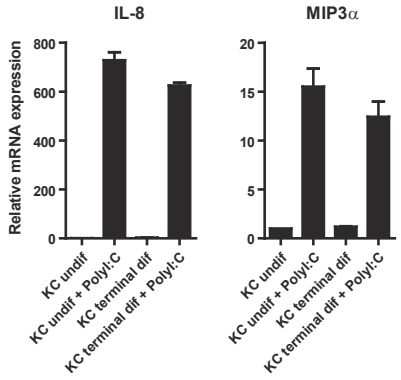

Figure S1 | Cytokine production by poly(I:C)-stimulated terminally differentiated keratinocytes. *IL-8* and *MIP3α* expression levels in unstimulated or poly(I:C)-stimulated uninfected KCs as examined by real-time PCR. KC were either left undifferentiated (undif) or terminally differentiated (terminal dif) with methylcellulose containing Ca^{2+}. Gene expression was normalized using *GAPDH*.

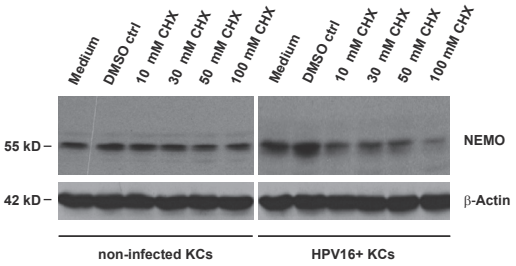

Figure S2 | NEMO degradation depends on the expression of UCHL1. NEMO degradation is enhanced in HPV16+ KCs but not in non-infected KCs. Monolayer cultures were treated with different concentrations of cycloheximide (CHX) for 24 hours. Whole cell extracts were analyzed by WB using antibodies against NEMO and β-actin (control for protein content).

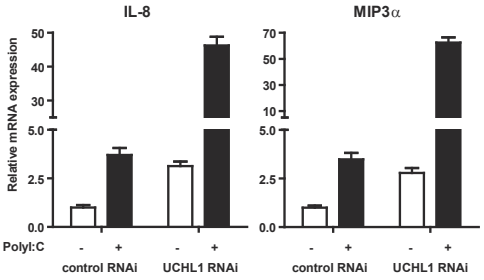

Figure S3 | Restored cytokine production after knock down of *UCHL1* by RNAi oligos. HPV16+ keratinocytes were transfected with non-targeting RNAi oligos and oligos targeting *UCHL1*. Cells were either left unstimulated, or were stimulated with poly(I:C) for 24 hrs. *IL-8*,and *MIP3α* mRNA expression was analyzed by qRT-PCR. Gene expression was normalized against *GAPDH* mRNA levels.

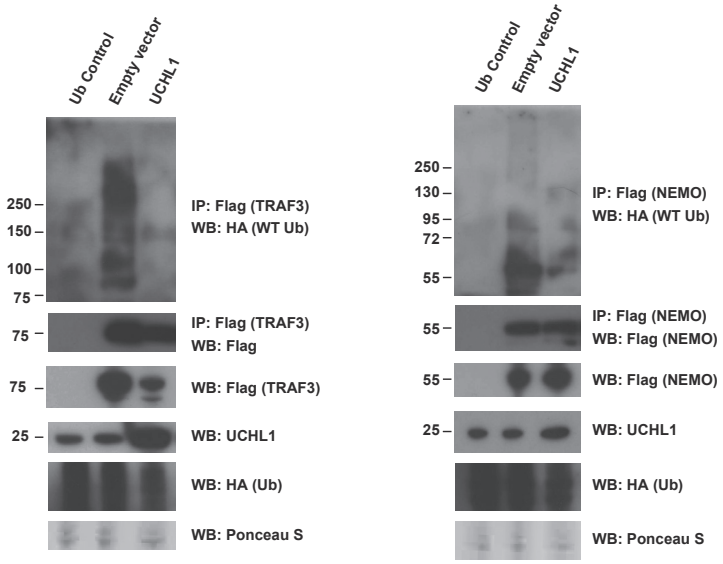

Figure S4 | TRAF3 and NEMO are deubiquitinated by UCHL1. HEK293T cells were co-transfected with HA-tagged wild-type ubiquitin (WT-Ub) only, with Flag-TRAF3 and HA-tagged wild-type ubiquitin (WT-Ub), and with Flag-TRAF3 and HA-tagged wild-type ubiquitin (WT-Ub) and UCHL1. A similar experiment was performed in which Flag-TRAF3 was replaced by Flag-NEMO (top panels). The bottom four panels show a WB analysis of Flag,Wt-Ub, and UCHL1 of non- immunoprecipitated lysate and a Ponceau S stained loading control for WB.

Table S1 | Enrichment of pathways between HPV-positive and uninfected keratinocytes as analyzed by Ingenuity Pathway Analysis (IPA).

Canonical pathway	***p*-value**
Purine Metabolism	1.15×10^{-5}
Oxidative Phosphorylation	6.26×10^{-5}
Protein Ubiquitination Pathway	6.69×10^{-5}
Graft-versus-Host Disease Signaling	5.35×10^{-4}
LXR/RXR Activation	7.55×10^{-4}
Mitochondrial Dysfunction	8.22×10^{-4}
Nucleotide Excision Repair Pathway	1.56×10^{-3}
Pyrimidine Metabolism	1.15×10^{-3}
NRF2-mediated Oxidative Stress Response	1.15×10^{-3}
Urea Cycle and Metabolism of Amino Groups	1.15×10^{-3}
Inositol Metabolism	1.15×10^{-3}
Glucocortocoid Receptor Signaling	8.41×10^{-3}
IL-10 Signaling	1.08×10^{-2}
Pentose Phosphate Pathway	1.34×10^{-2}
Glutathione Metabolism	1.43×10^{-2}
D-glutamine and D-glutamate Metabolism	1.46×10^{-2}
Hypoxia Signaling	1.88×10^{-2}
PPAR Signaling	1.94×10^{-2}
Arginine and Purine Metabolism	2.02×10^{-2}
Glutamate Metabolism	2.04×10^{-2}
Role of Cytokine in Mediating Communication between Immune Cells	2.2×10^{-2}
Aldosterone Signaling in Epithelial Cells	2.26×10^{-2}
Cardiac Hypertrophy Signaling	2.61×10^{-2}
Glycosphingolipid Biosynthesis- Neolactoseries	3.07×10^{-2}
Role of BRCA1 in DNA Damage Response	3.36×10^{-2}
Role of CHK Proteins in Cell Cycle Checkpoint Control	3.89×10^{-2}

SAMENVATTING (VOOR DE NIET-INGEWIJDE)

Het menselijk afweersysteem is een krachtig mechanisme om virusinfecties te bestrijden en te klaren. De groep van humane papillomavirussen (HPV) die geassocieerd zijn met het ontstaan van kanker, worden hoog-risico HPV (hrHPV) genoemd. In tegenstelling tot de meeste virussen waarmee we in contact komen, bijvoorbeeld verkoudheidsvirussen, die snel door het afweersysteem worden herkend en opgeruimd, kan een infectie met HPV ongemerkt maandenlang duren.

Om het organisme te beschermen bevatten de cellen van ons lichaam sensoren die in staat zijn om verschillende delen van virussen te herkennen. Wanneer een virus gedetecteerd wordt gaat er een signaal van de sensor naar de kern van de geïnfecteerde cel waarna deze chemische stofjes zal produceren die een directe antivirale werking hebben en stofjes die er voor zorgen dat de cellen van het afweersysteem naar het geïnfecteerde gebied komen zodat het virus vernietigd kan worden.

In hoofdstuk 2 lieten we zien dat hrHPV-geïnfecteerde cellen maar slecht in staat zijn om deze chemische stofjes te produceren. Verdere studies beschreven in hoofdstuk 3 toonden aan dat hrHPV op een slimme manier gebruikt maakt van een door de geïnfecteerde cel geproduceerd eiwit, genaamd UCHL1, om de door de virus-herkennende sensoren afgegeven signalen te onderdrukken zodat deze veel minder goed de kern van de cel bereiken waardoor de afweerreactie tegen het virus veel minder sterk is. Medicijnen die de werking van UCHL1 onderdrukken kunnen mogelijk baat bieden wanneer dit er toe leidt dat de afweerreactie sneller en beter op gang komt.

Tientallen jaren van onderzoek hebben aangetoond dat hrHPV verschillende typen kanker kan veroorzaken, waaronder baarmoederhalskanker. Tevens is in dezelfde periode aangetoond dat het afweersysteem het lichaam niet alleen goed kan beschermen tegen infecties met virussen maar ook tegen het ontstaan en de uitgroei van kanker cellen. De afweercellen die hier een belangrijke rol in spelen worden celdodende T lymfocyten (CTL) genoemd. Deze CTL worden vaak in grote hoeveelheden in baarmoederhalskankers aangetroffen en aangezien het kankergezwel behandeld moet worden is de vraag: waarom doen zij hun werk niet of niet goed genoeg? In hoofdstuk 5 van dit proefschrift hebben we één mogelijkheid bestudeerd. Het blijkt dat veel van de CTL die de tumor zijn binnengedrongen een eiwit tot expressie brengen dat PD-1 genoemd wordt. Signalen via dit eiwit kunnen leiden tot het afschakelen van CTL waardoor zij hun werk niet meer kunnen uitoefenen. Op dit moment is er een medicijn gemaakt dat de signalen via PD-1 kan blokkeren. Nieuwe studies zullen moeten uitmaken of dit medicijn ook baat biedt bij baarmoederhalskanker.

Tijdens de groei van een kankergezwel zullen regelmatig kankercellen zich verspreiden naar andere organen van het lichaam. Dit proces noemen we metastasering en de gezwellen die hieruit ontstaan worden metastasen genoemd. Het is een feit dat de meeste mensen dood gaan aan metastases. In hoofdstuk 6 laten we zien dat als de kankercellen van een patiënt het eiwit CXCR7, dat normaal betrokken is bij de migratie van afweercellen, tot expressie brengen zij veel vaker metastases ontwikkelen dan wanneer dit eiwit niet tot expressie komt.

In het kort werd in dit proefschrift onderzocht hoe het afweersysteem ontregeld is bij hrHPV infecties, hrHPV-geinduceerde kankers en hun metastases.

SUMMARY (IN PLAIN ENGLISH)

Human papillomaviruses (HPVs) that are associated with cancer are called high-risk HPVs (hrHPVs). The immune system is body's one of the most powerful system involves in clearing infections. Fortunately, most of the infections, for instance the viruses that cause common cold, are easily detected by the immune system and cleared. In contrast, HPV infection may remain undetected by the immune system for months. In Chapter 2 and Chapter 3 of this thesis, we examined how hrHPVs avoid host's immunity.

When cells are infected by the virus, the cells have sensors that detect various body parts of the virus. After detection of the virus, the cells produce certain chemicals that attracts the immune cells to help further so that the virus can be cleared. Our study in Chapter 2 showed that hrHPV infected cells minimally produce those chemicals, therefore, other immune cells are not attracted to come and to help clearing the infection. Our further work in the Chapter 3 identified that hrHPVs very smartly misuse infected cells own enzyme which is named as UCHL1 in order to suppress the immune responses of the cells. Drugs that may be able to inhibit the function of UCHL1 enzyme might be beneficial in clearing the hrHPV infection by host's own immunity.

Years of ongoing hrHPV infection is associated with various cancers including cervical cancer. Similar to infections, immune system is also very powerful in protecting tumor formation. The immune cells that are the main killers for tumors cells are named as cytotoxic T cells. However, in established cervical cancer tissue, these cytotoxic T cells cannot kill tumor cells. In Chapter 5 of this thesis, we examined the reasons why these tumoral cytotoxic T cells become dysfunctional. We observed that many of these cytotoxic T cells express a molecule named PD-1 which may render these cells to be malfunctioned against the killing of tumor cells.

Unresolved cancer spread from one organ to another which is called metastasis which is the main cause of death of cancer patients. Our studies in Chapter 6 where we used specimens from cervical cancer tissues identified that the patients whose cancer cells express an immune receptor called CXCR7 are more prone to have metastasis than those patients negative for CXCR7 expression.

Thus, this thesis examined the deregulation of immunity in early phages of hrHPV infection, in cervical cancer as well as during metastasis.

CURRICULUM VITAE

Rezaul Karim was born in Gaibandha, a northern district in Bangladesh. After completing higher secondary education from the Notre Dame College, Dhaka, Bangladesh, he enrolled in the Department of Microbiology and completed his Bachelor of Science (Honors) and Master of Science. He conducted his Master of Science thesis research on the diarrheagenic potential of environmental *Vibrio cholerae* strains non-O1 non – O139 strains using in vitro and rabbit model system supervised by Prof. Dr. Chowdhury R. Ahsan, Department of Microbiology, University of Dhaka. He then moved o the Netherlands in 2003 and completed his Master of Science in Infection and Immunity at the University of Utrecht. In Utrecht he first conducted 6 months research project on molecular diagnosis of influenza supervised by Dr. Rob Schuurman and Prof. Dr. Charles Boucher, Department of Virology, University Medical Center Utrecht. Afterwards, he joined the laboratory of Prof. Dr. Hans Clevers, Hubrecht Institute of Developmental Biology and Stem Cell Research, Utrecht for a year studying the role of BCL9 and Pygopus proteins in Wnt signaling under supervision of Dr. Wim de Lau. In 2005, he started his PhD research work at the Cancer Immunology section of the Department of Immunohematology and Blood Transfusion and at the Department of Human and Clinical Genetics, Leiden University Medical Center under the supervision of Prof. Dr. Sjoerd. H. van der Burg, Prof. Dr. Cornelis J.M. Melief, Dr. Judith M. Boer, Prof. Dr. Rienk Offringa, and Prof. Dr. Gert-Jan B. van Ommen. Final part of his PhD work was collaboratively conducted in the laboratory of Dr. Daniele Guardavaccaro, Hubrecht Institute of Developmental Biology and Stem Cell Research, Utrecht. His PhD research work has lead to this thesis. Since 2010, he has been working as a postdoctoral fellow in the laboratory Prof. Dr. Josef Penninger at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna, Austria on the molecular basis of cancer metastasis mainly using conditional knockout and genetic mouse models. He is married to Dr. Henrike E. Kos and they have two children, Norah (2010) and Ruben (2012).).

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