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General introduction

Adapted from:

Human papillomavirus targets crossroads in immune signaling

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INTRODUCTION

1.1 Human papillomaviruses

Human papillomaviruses (HPV) are small, non-enveloped viruses belonging to the *Papillomaviridae* family. The icosahedral virion encapsulates a double-stranded episomal DNA genome of 7 – 8 kb, comprising an early open reading frame (ORF), containing 6 non-structural early genes (E6, E7, E1, E2, E4 and E5), and a late ORF, containing 2 late genes (L2 and L1) that encode the capsid proteins. A non-coding long control region (LCR), between the L1 and E6 genes, contains regulatory elements that control replication and viral gene expression [1]. Over 180 HPV types have currently been identified. They are divided into genera α, β, γ, μ and ν, based on the nucleotide sequence of the L1 gene [2].

1.2 Clinical implications

HPV is widespread within all human populations and transmitted via the skin, including the genitalia. Diseases associated with HPV infection range from warts to cancers [3]. HPV types of the α genus (~40) infect cutaneous and mucosal epithelia. Based on their oncogenic potential, mucosal HPVs are classified as low-risk, associated with benign warts or epithelial lesions, or high-risk, that can cause oropharyngeal and anogenital malignancies, including cancers of the cervix, vulva, vagina, penis and anus. HPV types of the other genera infect cutaneous epithelium and are associated with non-melanoma skin cancer (β genus) or cutaneous papillomas and warts. Most HPV infections resolve spontaneously within one (70%) to two (90%) years [4], and in only <1% of cases malignancies develop. Still, HPV causes ~530,000 new cancer cases and ~275,000 deaths each year [5]. High-risk HPV (hrHPV) types are responsible for ~5% of all human cancers and 33% of all tumors induced by viruses. They are detected in 99.7% of cervical cancer cases, the second most common cancer in women, accounting for 20% of all cancer-associated deaths in women worldwide per year [6, 7]. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 59, 69, 73 and 82 have been detected in cervical carcinomas, but HPV16 and 18 are by far the most prevalent types responsible for ~50% and ~20% of all cases, respectively [8, 9].

1.3 The viral life cycle

HPVs exclusively infect keratinocytes (KCs) of the basal layer of the epidermis and mucosal epithelia, which they reach via micro-wounds and abrasions. Binding of the L1 protein of HPV to heparan sulfate proteoglycans at the surface of KCs induces endocytosis of the virion. Subsequently, the capsid disassembles following acidification of the endosome and then the viral episome, still associated with L2, travels via the Golgi apparatus and ER to the nucleus [10] where low levels of viral early proteins are produced that reside mainly in the nucleus [11]. E1 and E2 initiate episome replication and, together with the host DNA replication machinery, maintain a low episome copy-number of 50 – 100 per cell [12]. Furthermore, E6 and E7 are produced to prevent cell growth arrest and apoptosis and delay differentiation, by inactivating p53 and the retinoblastoma protein (pRB). This induces a proliferative, non-differentiating state of the infected KC, resulting in lateral cell division. As the infected KC differentiates and migrates through the suprabasal layers of the epithelium, the expression of all viral genes is induced to enhance viral episome replication, which reaches high copy-numbers of hundreds to thousands per cell. In the higher layers of the epithelium the production of the late proteins L1 and L2, together forming the viral capsid, is induced and virion assembly takes place. With the rupture and shedding of the matured KC the viral particles are released [13].

1.4 Malignant transformation

Sometimes, for yet unknown reasons, hrHPV genomes can spontaneously integrate into the host genome. When this happens, the E6 and E7 oncogenes are fully integrated whereas the E2 gene is not. Abrogation of E2 expression releases the tight regulation of E6 and E7 expression. The newly transformed cells stably express E6, which binds to p53 and recruits the E3 ligase E6AP to target p53 for proteasomal degradation, as well as E7, which recruits the E3 ligase cullin 2 to target pRb for proteasomal degradation. The loss of these tumor suppressors results in uncontrollable cell growth, host genome mutations and inhibition of apoptosis, ultimately leading to cancer formation [1, 13, 14].

2. HPV immune evasion strategies

High-risk HPV infections can last up to two years despite viral activity in keratinocytes, indicating that hrHPV has developed mechanisms to effectively evade or suppress the hosts innate and/or adaptive immune response. Indeed, several studies on the spontaneous immune response to HPV have shown that HPV-specific cellular immunity develops quite late during persistent HPV infections and often is of dubious quality in people with progressive infections [15].

Viral persistence may be linked to the life cycle of HPV since HPV does not cause viremia, cell death, or cell lysis, and the life-cycle takes place within the boundary of the *lamina basalis*, away from dermal immune cells. Thus, spontaneous contact between the immune system and the virus is minimal and inflammatory responses are not readily elicited. Langerhans cells residing within the epidermis can sense viral presence, but HPV counteracts their recruitment by interfering with the production of immune attractants. After the infection is established, the virus produces only low quantities of viral proteins that mainly reside in the nucleus of the cells. Besides these passive mechanisms to evade the immune system, hrHPV also actively interferes with innate and adaptive immune mechanisms.

2.1 Viral recognition by keratinocytes

Keratinocytes are well equipped to sense pathogens. Basal KCs express pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RNA helicases, to recognize pathogen-associated molecular patterns (PAMPs) on viruses and microbes. PRR ligation leads to activation of inflammatory and proliferative signaling cascades and subsequent production of pro-inflammatory cytokines that can induce innate and adaptive immune responses. KCs express TLR 1, 2, 3, 5 and 6 on the cell-surface and the nucleic acid-sensing TLR3 in endosomes. TLR7 and TLR8 are not expressed, but TLR7 expression can be induced upon TLR3 ligation [16]. The expression of TLR4 and TLR9 in basal KCs is still under debate, but TLR9 expression can be induced after terminal differentiation [17]. Cytosolically, KCs express the RNA helicases retinoic acid-inducible gene I (RIG-I; DDX58) and melanoma differentiation-associated protein 5 (MDA5; IFIH1) [18], and the dsDNA sensors gamma-interferon-inducible 16 (IFI16) and absent in

melanoma 2 (AIM2) [19].

Although the vesicle-mediated entry mechanism used by HPV may hide the virus from recognition by cytoplasmic DNA sensors, KCs can produce type I interferon (IFN) and pro-inflammatory cytokines upon viral entry and, therefore, do recognize HPV [17]. Indeed, the episome contains CpG motifs that can be recognized by TLR9 [20] and the viral capsid itself is a potential PAMP. Whether HPV interferes with the expression of TLRs, RIG-I or MDA5 in HPV episome-containing KCs is still under debate [17, 18, 21], and while *TLR9* expression and function was shown to be abolished in KCs that overexpressed HPV16 E6 and E7 [20], by an E7-induced recruitment of a NFκB1, ERα and HDAC1 inhibitory complex to the TLR9 promotor [22], others concluded that E6 nor E7 influenced TLR9 expression or function [23]. The DNA sensor AIM2 is strongly expressed in HPV16-infected skin lesions, whereas IFI16 expression is not elevated [19]. Hence, it is not yet clear if HPV affects the expression of virus sensory molecules on KCs.

2.2 HPV influences innate immune signaling

Keratinocytes produce type I IFNs and pro-inflammatory cytokines upon PRR ligation through signaling via interferon regulatory factor (IRF) and nuclear factor of kappa-light-chain-enhancer of activated B cells (NFκB) activating pathways. Type I IFNs (mainly IFNα (13 subtypes) and IFNβ, but also IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ) stimulate cells to express genes inducing an anti-viral state. They can also stimulate dendritic cells and as such act as a bridge between innate and adaptive immunity [24-26]. Pro-inflammatory cytokines are chemoattractants for immune cells and regulate cell migration, activation, polarization and proliferation. Several genome-wide transcription studies reported that hrHPV types 16, 18 and 31 influence – mainly reduce – basal, TLR3-induced cytokine expression, and type I IFN-induced interferon-stimulated gene (ISG) expression [18, 27-29], indicating that hrHPV affects PRR- and type I IFN-induced signaling pathways.

2.2.1 The effect of HPV on the IRF signaling pathway

All TLRs, except TLR3, convey their signals via the adapter molecule MyD88. This induces the IRAK complex (consisting of IRAK1, 2 and 4) to recruit TRAF3, which stimulates IKKα to phosphorylate IRF7. TLR3 and 4 signal via

TRIF, cytosolic RNA sensors via MAVS, and cytosolic DNA sensors signal via the adaptor molecule STING to activate TRAF3, which then induces the TBK1-IKKε complex to phosphorylate IRF3. Phosphorylated IRF3 and IRF7 homo-dimerize and translocate to the nucleus where production of type I IFNs is initiated. Furthermore, PRR ligation can result in IRF1 activation (Figure 1).

HrHPV influences type I IFN production by interfering at several points in the signaling cascade. Its E2 proteins reduce the expression of *STING* and *IFNκ* [30], the latter of which its expression is also reduced by E6 [21, 31]. HPV16, but not HPV18, E6 protein binds to IRF3 and, thereby, may prevent its transcriptional activity [32]. E7 blocks *IFNβ* transcription by binding to IRF1 and recruiting histone deacetylases (HDACs) to the *IFNβ* promotor site [33, 34]. In contrast, E5 enhances *IFNβ* and *IRF1* expression [35].

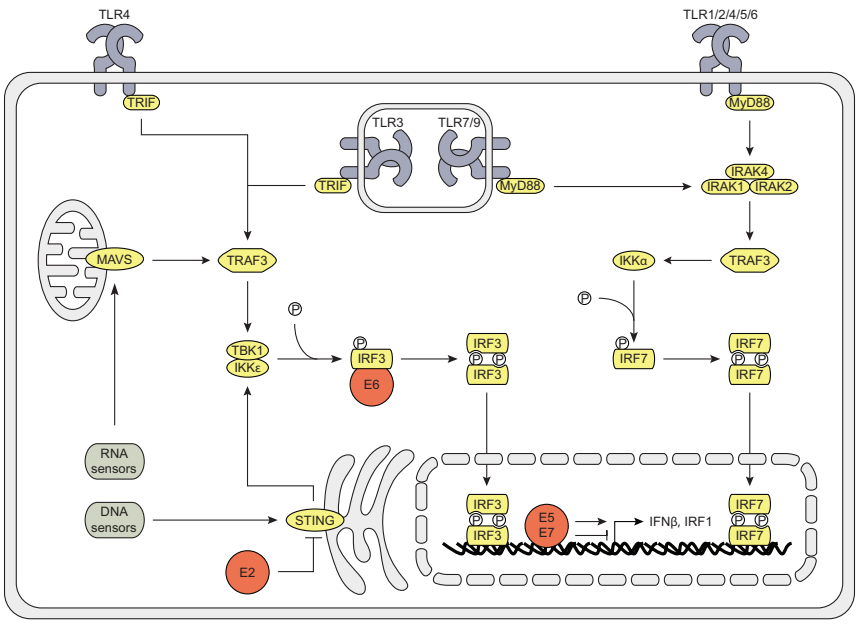

Figure 1: The effects of hrHPV on IRF signaling
Schematic representation of the effects of hrHPV on IRF signaling. All TLRs, except TLR3, activate IRF7 via signaling through MyD88, the IRAK complex, TRAF3 and IKKα. TLR3 and 4 signal via TRIF, cytosolic RNA sensors through MAVS and cytosolic DNA sensors via STING activate IRF3 through TRAF3, TBK1 and IKKε. Activated IRFs dimerize, translocate to the nucleus and initiate gene transcription. HPV utilizes its encoded E proteins (red) to interfere with these signaling pathways.

2.2.2 The effects of HPV on IFNAR signaling

The PRR-induced type I IFNs IFNα and IFNβ are secreted and can induce IFN-stimulated gene (ISG) expression in the infected cell itself but also in their uninfected neighbors. IFNα and IFNβ bind to the heterodimeric transmembrane IFNα/β receptor (IFNAR), composed of the IFNAR1 and IFNAR2 subunits. The IFNAR activates the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which recruit and phosphorylate STAT1 and STAT2, causing them to hetero-dimerize, bind IRF9, thereby forming the IFN-stimulated gene factor 3 (ISGF3) complex, and translocate to the nucleus. ISGF3 binds to IFN-stimulated response elements (ISREs) on the DNA and activates ISG transcription. IFNAR ligation can also lead to STAT1 homo-dimerization. STAT1 homo-dimers translocate to the nucleus and bind to γ-activated sequences (GAS) on the DNA, thereby activating ISG transcription more associated with IFNγ signaling (Figure 2) [25, 36].

HrHPV also interferes with IFNAR signaling. HPV18 E6 can bind to TYK2 in order to hamper phosphorylation of STAT1 and STAT2 [37]. E6, and to a lesser extend E7, of the hrHPV types 16 and 31 were shown to impair STAT1 transcription and translation, and binding of STAT1 to the ISRE [27, 29, 38]. However, although hrHPV represses STAT1 protein levels, the IFNβ-induced STAT1 signal cascade is not affected by hrHPV, as phosphorylation of STAT1 still occurs [38]. Expression of STAT2 and IRF9 are not affected, but E7 can interact with cytosolic IRF9, preventing IRF9 to translocate to the nucleus with as a consequence impairment of ISGF3 complex formation [39, 40].

2.2.3 The effect of HPV on the NFκB signaling pathway

PRRs also induce cytokine production through signaling via TRIF, MAVS, STING and the IRAK complex, which leads to the K63-linked poly-ubiquitination of TRAF6. The TAB1-TAB2-TAK1 complex and the IKK complex (consisting of NEMO, IKKα and IKKβ) bind to the poly-ubiquitin chain on TRAF6, resulting in the phosphorylation of IKKβ by TAK1. Activated IKKβ then phosphorylates IκBα, leading to the SCF-βTrCP-mediated K48-linked poly-ubiquitination of IκBα and its subsequent degradation. This releases the NFκB1 complex (consisting of RelA and p50) and allows it to translocate to the nucleus where it is further modified to induce DNA binding and transcriptional activation (Figure 3) [26, 41].

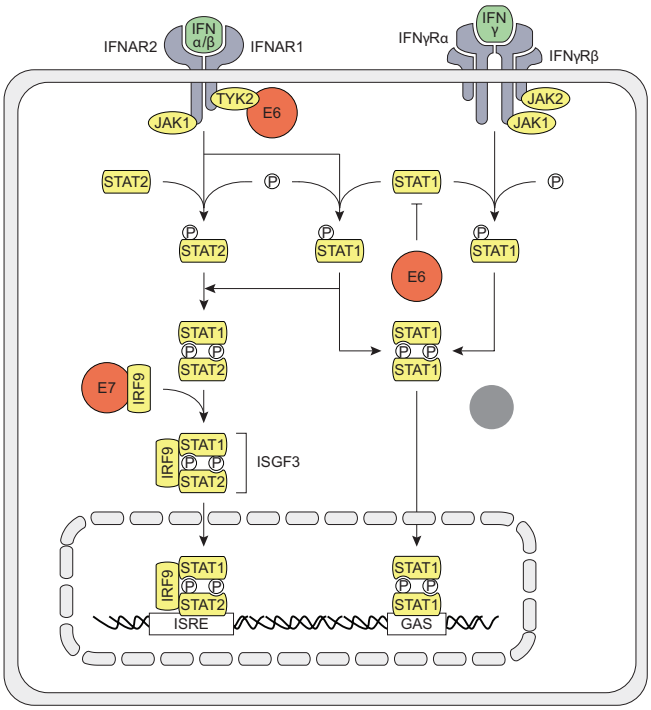

Figure 2: The effects of hrHPV on IFNAR and IFNγR signaling
Schematic representation of the effects of hrHPV on IFNAR and IFNγR signaling. Type I IFN binding to the IFNAR leads to signaling via JAK1 and TYK2 to activate STAT1 and STAT2. STAT1 and STAT2 heterodimerize and recruit IRF9, forming the ISGF3 complex, which translocates to the nucleus, binds to ISREs and initiates ISG transcription. Activated STAT1 can also homodimerize, translocate to the nucleus, bind to GAS and initiate ISG transcription. Type II IFN binding to the IFNγR results in the activation of JAK1 and JAK2 and recruitment and phosphorylation of STAT1, which homo-dimerizes, translocates to the nucleus, binds to GAS on the DNA and initiates ISG transcription. HPV proteins (red) interfere with both IFNAR and IFNγR signaling by decreasing STAT1 levels, and hampering TYK2 and IRF9.

By the use of several different model systems hrHPV or its individual proteins have been shown to affect the PRR-induced signaling cascade that leads to NFκB nuclear translocation and to impair the function of NFκB within the nucleus. HrHPV upregulates the NFκB family members RelA, c-Rel, and the precursor proteins p105 and p100, which are processed into p50 and p52, respectively, and sequesters these proteins in the cytoplasm [29, 42-45]. The last NFκB family member, RelB, is not reported to be regulated by HPV.

Within the nucleus, E6 reduces NFκB RelA-dependent transcriptional activity [46], by binding to the C/H1, C/H3 and C terminal domains of CBP/p300 [47, 48], thereby competing with RelA and SRC1, which bind the C/H1 and C terminal domain of CBP/p300, respectively [49]. P/CAF can still bind to the C/H3 domain of CBP/p300 in presence of E6, but P/CAF cannot acetylate NFκB since E7 binds to, and thereby blocks, the HAT domain of P/CAF [49]. E7 blocks NFκB DNA binding activity [34] and competes with E2 for binding the C/H1 domain of p300/CBP, thereby hampering E2 transactivation [50]. In contrast, E2 binds to p300/CBP [51, 52] and increases NFκB signaling by enhancing RelA expression and transcriptional activation upon TNFα treatment [44].

2.2.4 The effect on the inflammasome pathway

It is not clear whether the inflammasome pathway is important in the protection against HPV. However, recently it was reported that the production of IL1β, a cytokine that is secreted upon cleavage of pro-IL1β by inflammasome-activated caspase1, is impaired. HPV E6 binds to E6-AP and p53 and this complex induces the inflammasome-independent proteasome-mediated degradation of pro-IL1β and as such hampers IL1β formation [53], indicating that hrHPV may suppress immunity by interference with post-translational processes.

2.3 HPV suppresses the action of KCs to secondary immune signals

Cells of the adaptive immune system, in particular T cells, are activated by antigen-presenting cells (APCs) in the lymph nodes and migrate to infected sites. They produce cytokines and express ligands that can activate signaling cascades in the KC involved in survival and pro-inflammatory cytokine production, leading to killing of KCs and in parallel the reinforcement of

adaptive immunity. Despite the infiltration of adaptive immune effector cells the persistence of hrHPV-infected sites suggests that hrHPV has evolved mechanisms to resist this attack. Especially $CD4^+$ T helper 1 (Th1) cells are important in controlling hrHPV infections. However, even vaccines that boost viral Th1 immunity during chronic infection are only partially successful [54]. Th1 cells produce IFNγ and TNFα, and express CD40L, which induce cytokine production and proliferative changes in KCs.

2.3.1 The effect of HPV on the TNFα-activated NFκB signaling pathway

TNFα is the ligand for both the TNFα receptor 1 (TNFR1) and TNFR2. TNFR1 activates canonical NFκB1 by recruiting and activating TRADD, leading to the formation of a complex consisting of RIP1,

TRAF2 or 5, and cIAP1 or 2. cIAP1/2 is ubiquitinated with a K63-linked poly-ubiquitin chain to which the LUBAC complex (consisting of Sharpin, HOIP and HOIL1) binds. RIP1 is ubiquitinated with both K63-linked and linear poly-ubiquitin chains. The TAB1-TAB2-TAK1 complex binds to the K63-linked poly-ubiquitin chain and phosphorylates the IKK complex that binds to the linear poly-ubiquitin chain of RIP1, leading to NFκB1 release through IKKβ-induced SCF-βTrCP-mediated degradation of IκBα. TNFR2 activates the non-canonical NFκB2 pathway by recruiting TRAF2/5, cIAP1/2 and TRAF3, resulting in TRAF3 degradation. This abrogates TRAF3-induced NIK degradation, causing NIK to accumulate and activate IKKα. IKKα phosphorylates the p100 NFκB precursor protein of the NFκB2 complex, which further consists of RelB. This induces SCF-βTrCP to ubiquitinate p100 with a K48-linked poly-ubiquitin chain, leading to the proteosomal processing of p100 into p52, and the subsequent nuclear translocation of the p52-RelB dimer (Figure 3).

HPV interferes with these cascades in a similar way as it attenuates PRR-induced NFκB. Additionally, E6 binds to the C terminus of TNFR1 [55], and the N terminus of the death effector domains (DEDs) of FADD, which accelerates the degradation of FADD [56], thereby hampering the induction of apoptosis. E6 does not bind to the TRADD adaptor molecule [56]. Furthermore, E7 binds to the IKK complex and attenuates TNFα-induced kinase activity of IKKα and IKKβ, which hampers IκBα phosphorylation and degradation, and subsequent NFκB nuclear translocation [46]. In contrast to E6 and E7, E2 stimulates TNFα-induced, but not IL1-induced, NFκB signaling [44, 57], by directly interacting

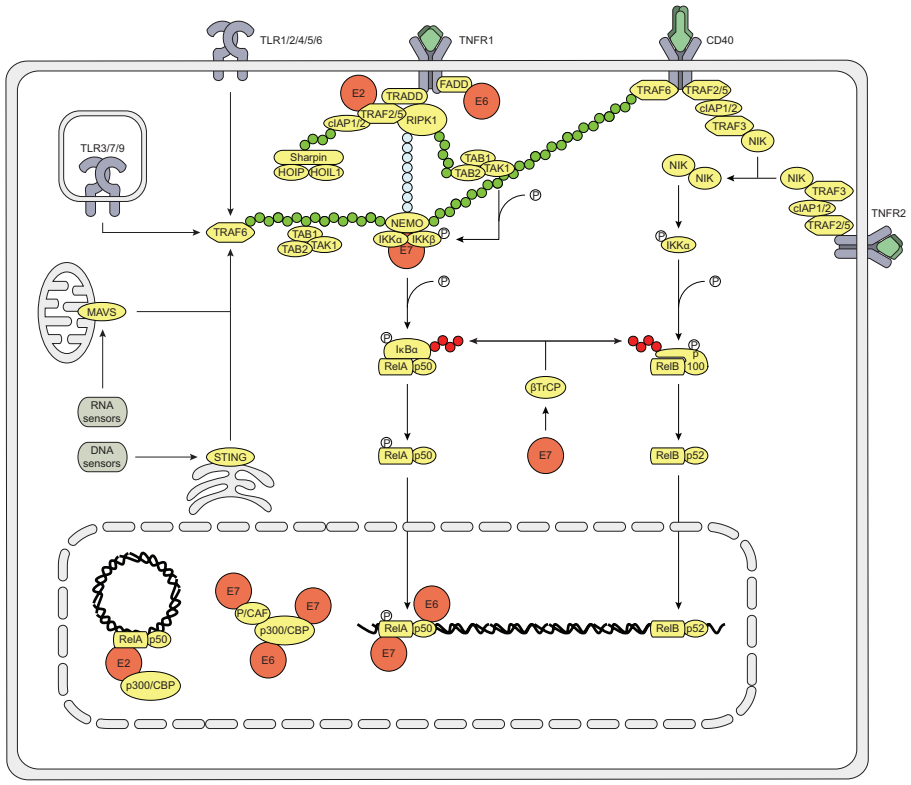

Figure 3: The effects of hrHPV on NFκB signaling

Schematic representation of the effects of hrHPV on NFκB signaling. The canonical NFκB1 pathway is activated by PRRs and CD40 through TRAF6 and TNFR1 through RIP1. Poly-ubiquitination of TRAF6 and RIP1 recruits the TAB1-TAB2-TAK1 and IKK complexes resulting in the phosphorylation of IKKβ by TAK1. IKKβ phosphorylates IκBα, which is then ubiquitinated by SCF-βTrCP and subsequently degraded, and thereby releases the NFκB1 complex to translocate to the nucleus. CD40 and TNFR2 initiate non-canonical NFκB2 signaling by recruitment of TRAF2/5, cIAP1/2 and TRAF3 to the respective receptor, leading to TRAF3 degradation. This causes NIK to accumulate and activate IKKα to phosphorylate p100. This induces SCF-βTrCP to ubiquitinate p100, leading to the proteosomal processing of p100 into p52, and the subsequent nuclear translocation of NFκB2. In the nucleus NFκB binds to the DNA and is aided by coactivators to initiate gene transcription. HPV utilizes its encoded E proteins (red) to interfere with NFκB1 signaling at multiple positions in the pathway. Green circles indicate K63-linked poly-ubiquitin chains, red circles indicate K48-linked poly-ubiquitin chains, and blue circles indicate linear poly-ubiquitin chains.

with TRAF5 and TRAF6, but not TRAF2, thereby stimulating K63-linked ubiquitination of TRAF5 [57].

2.3.2 The effects of HPV on IFNγR signaling

Ligation of the IFNγR with type II IFN results in the activation of JAK1 and JAK2 and recruitment and phosphorylation of STAT1, which homo-dimerizes, translocates to the nucleus, binds to GAS on the DNA and initiates ISG transcription (Figure 2). The effects of hrHPV on the IFNγ-signaling pathway might be explained by the repressed STAT1 expression and protein levels in HPV infected cells, albeit that STAT1 phosphorylation still is intact [38].

2.4 HrHPV influences MHC surface expression and peptide presentation

The attack of virus-infected cells by T cells is a highly effective and specific mechanism to prevent the production and spread of virus particles. T cells recognize cells when viral protein-derived peptides are presented in the context of MHC molecules. Literature shows that primary KCs constitute excellent targets for antigen-specific cytotoxic T lymphocytes (CTLs) if their cognate peptide is presented on the KCs cell surface [58]. The overexpression of E5 [59] or E7 [60], however, makes cells more resistant to CTL-mediated lysis. E5 and E7 both reduce MHC-I surface expression, but act on different levels (Figure 4). E7 reduces MHC-I gene expression by physically associating with a putative RXRbeta binding motif (GGTCA) of the proximal promoter of MHC-I genes and recruiting HDAC1, 2 and 8 to this promoter site, leading to repressed chromatin activation. Indeed, E7 knock-down in Caski cells released HDAC1 and 2 from the MHC class-I promoter, and increased histone acetylation and MHC-I expression [60-64]. Furthermore, E7 represses the LMP2 and TAP1 promotors [61, 62], two important proteins involved in peptide production and transportation, respectively. E7 also reduces IRF1 expression by suppression of IFNγ-induced STAT1-Tyr701 phosphorylation, repressing IFNγ-mediated upregulation of MHC-I expression via the JAK1/JAK2/STAT1/IRF-1 signal transduction pathway [65, 66]. E5 does not influence MHC-I synthesis, but reduces MHC-I surface expression [65] by retaining MHC-I in the Golgi complex via interaction of di-leucine motifs (LL1 and LL3) localized in the N-terminal helical transmembrane (TM1) region of the protein [67]. This E5 – MHC-I interaction is not haplotype specific, suggesting that E5 can hamper

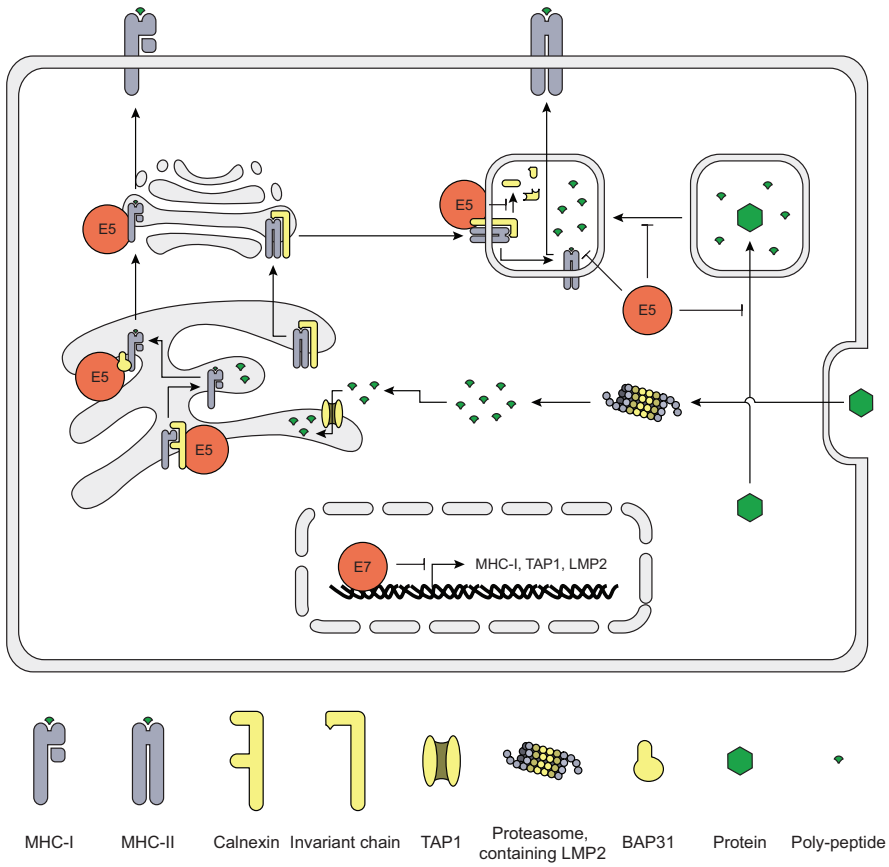

Figure 4: The effects of hrHPV on antigen presentation

Schematic representation of the effects of hrHPV on antigen presentation. The proteasome processes proteins into peptides, which are transported into the ER via TAP1. Aided by several chaperone proteins, MHC-I is folded and loaded with peptide after which it exits the ER to travel via the Golgi apparatus to the plasma membrane were the peptides are presented to T cells. HPV proteins (red) attenuate gene expression of critical components of this pathway as well as actively retains MHC-I in the ER and Golgi apparatus. MHC-II forms in the ER and complexes with the invariant chain. The complex travels via the ER and Golgi apparatus to lysosomes where the invariant chain is degraded and MHC-II is loaded with processed peptides from endocytosed proteins. Loaded MHC-II then travels to the plasma membrane to present the peptides. Upon IFNγ stimulation, HPV E5 (red) blocks invariant chain degradation and peptide loading, as well as inhibits endosome acidification and maturation.

all MHC-I-dependent antigen presentation [68]. Moreover, binding of the TM1 domain of E5 to the ER chaperone Calnexin retains MHC-I in the ER [69], and down-regulates surface expression of CD1d, a sentinel protein in bridging innate and adaptive immunity [70]. Furthermore, via its C-terminus E5 can bind the B-cell-associated protein 31 (BAP31) [71], a protein involved in the exit of peptide-loaded MHC-I from the ER [72]. Interestingly, E5 selectively downregulates the surface expression of HLA-A and -B, but not that of HLA-C and HLA-E [65]. Under normal conditions expression of HLA class II is not affected but upon IFNγ stimulation E5 does abrogate MHC-II surface expression and blocks peptide-loading of MHC-II and invariant chain degradation [73], by inhibiting endosome acidification [74] or perturbing trafficking from early to late endocytic structures [75].

SCOPE OF THIS THESIS

In this thesis we examined how hrHPV interferes with innate and adaptive immune signaling in keratinocytes. It is clear that hrHPV invests heavily in 1) preventing infected cells to adapt an anti-viral state, 2) suppressing the production of cytokines that can induce the attraction of adaptive immune cells which may control HPV infection, and 3) perturbating the expression of HLA class I and II molecules making the infected cells less visible to the adaptive immune system. Until now, studies focused specifically on the effects of hrHPVs' early proteins. In the present studies, we show that hrHPV also exploits cellular proteins to intervene with innate and adaptive immune signaling. We used a cell culture system that resembles the natural infection of KCs with hrHPV as close as possible. Primary basal layer KCs of foreskin, vaginal and cervical origin stably expressing full HPV16 or HPV18 episomes following electroporation were studied. These HPV-positive KCs mimic HPV infection *in vivo* as they undergo the entire differentiation-dependent HPV life cycle, documented by episome amplification, late gene expression and virus production, when cultured in organotypic raft cultures [76-78]. These raft cultures produce infectious authentic HPV virions, which we used to infect basal primary KCs.

In **chapter 2**, the impact of hrHPV presence in KCs on the production of type I IFNs and pro-inflammatory cytokines after PRR ligation was assessed. HrHPV presence attenuated pro-inflammatory cytokine production, hampered RelA and IRF3 phosphorylation, and decreased NEMO protein levels. HrHPV upregulated the expression of UCHL1, which impaired IRF3 phosphorylation by removing K63-linked polyubiquitin chains from TRAF3, thereby impairing TBK1 binding to TRAF3. Furthermore, UCHL1 hampered RelA phosphorylation by affecting TRAF6 ubiquitination and inducing degradation of NEMO.

Chapter 3 focused on the reaction of keratinocytes to CD40 ligation and the impact of hrHPV herein. Keratinocytes reacted very coordinately to CD40 ligation, predominantly expressing genes involved in leukocyte migration, cell-to-cell signaling and interaction, as well as cell death and survival. HrHPV presence did not affect the gene expression profile of CD40 stimulated KCs, but attenuated the extent of the response, resulting in the impairment of the attraction of PBMCs.

In **chapter 4**, the impact of hrHPV presence in KCs on pro-inflammatory cytokine production after IFNγR and TNFR ligation was studied. Pro-inflammatory cytokine production induced by IFNγ, TNFα or their combination was impaired when hrHPV was present in the KCs. HrHPV attenuated RelA acetylation by upregulating the expression of IFRD1 in an EGFR-dependent way. The effects of hrHPV on the cytokine expression in KCs could partially be overcome by treatment with the EGFR antibody cetuximab and the HDAC1/3-specific inhibitor entinostat. Notably, these treatments also enhanced Poly(I:C)-induced cytokine expression.

In **chapter 5**, the impact of hrHPV on the effects of IFNγ, TNFα and their combination on the proliferation of keratinocytes was studied, as these cytokines were shown to have growth inhibitory effects on KCs. HrHPV rendered KCs resistant to the growth inhibitory effects of these cytokines by counteracting the IFNγ-induced arrest in cell proliferation via downregulation of the anti-proliferative gene *IFITM1*.

Finally, **chapter 6** presents a general discussion and the conclusions of the findings of this thesis.

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