Human papillomavirus targets crossroads in immune signaling

Tummers, Bart

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General discussion

GENERAL DISCUSSION

Keratinocytes are well equipped to recognize and react to invading pathogens, and hrHPV is no exception to this. However, hrHPV initiates several immune evasion mechanisms soon after infecting the KC. The virus interferes with the innate immune response by affecting several signaling pathways that otherwise would prompt anti-viral mechanisms in the host cell. Furthermore, hrHPV interferes with the production of cytokines that are involved in the attraction of immune cells to the infected epithelium. In addition, the virus hides itself from the immune system by suppressing the antigen presentation machinery normally allowing infected cells to be recognized by adaptive immune cells and, if this is not successful, hrHPV still employs means to hamper the response of KC's to signals from the effector molecules used by adaptive immune cells to exert their antiviral function. In this thesis we show that hrHPV attenuates innate immune signaling (**Chapter 2**) and CD40-mediated (**Chapter 3**) and IFNγ and/or TNFα-induced (**Chapter 4**) adaptive immune signaling. For this hrHPV exploits the cellular proteins UCHL1 (**Chapter 2**) and IFRD1 (**Chapter 4**) that act on multiple points in the IRF and NFκB signaling pathways. Moreover, hrHPV downregulates cellular IFITM1 to resist the growth inhibitory effects of IFNγ and/or TNFα (**Chapter 5**). Taken together, our data provide important new insights on how the small hrHPV can persist in the face of host immunity.

HPV exploits cellular proteins to alter canonical NFκB signaling

The canonical NFκB pathway is attacked by hrHPV at multiple positions in the signaling cascade downstream of immune receptors. This indicates that suppression of the NFκB pathway forms a very important target for the virus and implies that this pathway normally would allow the host to resist viral infection. There are several early proteins involved in this process (see Chapter 1). The observations made in this thesis using hrHPV episome-baring KCs revealed that hrHPV exploits the cellular proteins UCHL1 and IFRD1 to interfere with NFκB signaling.

We showed that HPV-induced UCHL1 attenuates PRR-induced type I IFN and pro-inflammatory cytokine expression (Chapter 2). UCHL1 hampered the

IRF pathway by interacting with and deubiquitinating K63-linked polyubiquitin chains from TRAF3, resulting in reduced TBK1 – TRAF3 interaction, IRF3 phosphorylation and *IFNβ* expression (Figure 1). PRR-induced NFκB signaling was also attenuated through binding of UCHL1 to TRAF6, thereby influencing the Ub status of TRAF6 (Figure 2). Furthermore, UCHL1 exacerbated NEMO degradation and UCHL1 can prevent IκBα ubiquitination [1].

That UCHL1 binds and affects the ubiquitination status of TRAF3 and 6 implies that UCHL1 may influence other TRAF proteins as well. Indeed, co-immunoprecipitation (co-IP) experiments of UCHL1 and TRAF1-6 in HEK293T cells showed that UCHL1 can bind to all TRAFs (Tummers, *Unpublished data*) and might therefore be a regulator of TRAF ubiquitination and thus function. Furthermore, our co-IP experiments showed that UCHL1 binds to RIP1. In line with this, UCHL1 may influence adaptive immunity-induced canonical and non-canonical NFκB signaling, since the TRAF proteins and RIP1 mediate these pathways [2]. Indeed, knock-down of UCHL1 in HPV-episome expressing KCs enhanced pro-inflammatory cytokine expression upon IFNγ and/or TNFα or CD40L (Tummers, *Unpublished data*). Furthermore, although the two proteins do not co-immunoprecipitate, UCHL1 mediated the degradation of NEMO (Chapter 2). How UCHL1 does this is currently unknown, but, as TRAF6 facilitates the phosphorylation of the IKK complex by TAB1-TAB2-TAK1, one could speculate that UCHL1 is in close enough proximity to NEMO to facilitate its degradation, suggesting that UCHL1 may have a variety of cellular protein targets.

EGFR activation on epithelial cells has been shown to result in a decreased production of pro-inflammatory cytokines [3-5]. HrHPV upregulates EGFR gene and surface expression via the E5, E6 and E7 proteins (Chapter 4 and [6]), and enhances EGFR signaling via E5 and E6 [7-9]. Blocking the EGFR on our HPV+ KCs using the clinically used anti-EGFR antibody cetuximab augmented the production of IFNγ and TNFα-induced production of pro-inflammatory cytokines, indicating that by elevating EGFR levels and signaling HPV may hamper cytokine production (Chapter 4). Via EGFR signaling through mTOR, RAF and/or MEK1, HPV increased the expression of IFRD1, which mediates RelA K310 deacetylation by HDAC1/3 [10] and, thereby, attenuates the transcriptional activity of NFκB1 (Chapter 4 and Figure 6). IFRD1 knock-down

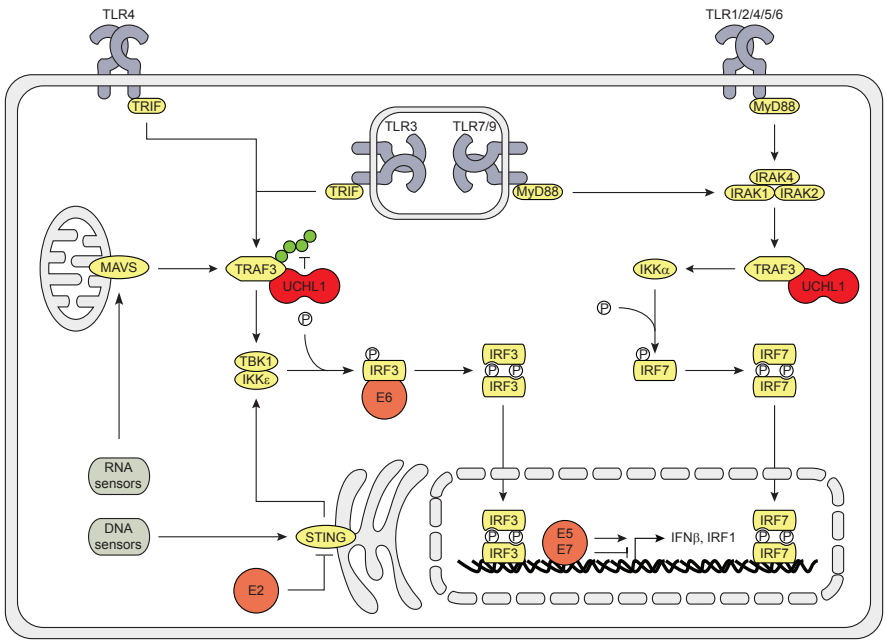

Figure 1: The effects of hrHPV on IRF signaling
Schematic representation of the effects of hrHPV on IRF signaling. All TLRs, except TLR3, activate IRF7 via signaling through MyD88, the IRAK complex, TRAF3 and IKKα. TLR3 and 4 signal via TRIF, cytosolic RNA sensors through MAVS and cytosolic DNA sensors via STING activate IRF3 through TRAF3, TBK1 and IKKε. Activated IRFs dimerize, translocate to the nucleus and initiate gene transcription. HPV utilizes its own encoded E proteins (red) as well as exploits the cellular protein UCHL1 (red) to interfere with these signaling pathways. Green circles on TRAF3 indicate K63-linked poly-ubiquitin chains.

experiments in HPV+ KCs indeed showed that basal RelA acetylation was restored and basal signaling and signaling induced by Poly(I:C), TNFα and the combination of IFNγ and TNFα resulted in higher cytokine expression levels in cells in which IFRD1 was knocked-down (Chapter 4). Interestingly, after IFNγ stimulation alone cytokine expression levels were also higher in IFRD1 knock-down HPV+ KCs, suggesting that IFRD1 may also affect the transcriptional activity of STAT1 and/or IRF1. If IFRD1 can regulate transcriptional activity of transcription factors other than NFκB, HPV could deregulate a whole network of cellular genes by simply exploiting one cellular protein.

Interestingly, E2 may promote canonical NFκB signaling [11-13]. It may form an E2-NFκB-p300/CBP transcriptional repressor complex on the LCR of the episome and as such regulates episome transcription which is required for the virus to sustain a low profile. However, as luciferase assays show that the E2 protein renders NFκB more active [13], the virus thus may prompt E2-mediated NFκB-induced pro-inflammatory cytokine production and immune cell attraction. This indicates that the virus needs additional mechanisms in order to regulate the episome while keeping pro-inflammatory cytokine expression in check during infection. The combined expression of E2, UCHL1 and IFRD1 during an infection might form a perfect cocktail to allow hrHPV to regulate its episome while suppressing KCs pro-inflammatory cytokine production.

HPV allows signaling to the non-canonical NFκB pathway

IFNγ and TNFα are known to synergistically affect gene expression, and also in KCs pro-inflammatory cytokine expression is synergistically higher than expression induced by IFNγ or TNFα alone (Chapter 4). Still, hrHPV attenuates IFNγ and/or TNFα-induced pro-inflammatory cytokine expression and the attraction of PBMCs to KCs that have been stimulated with the combination of IFNγ and TNFα. Furthermore, exposure of hrHPV-infected KCs to IFNγ and TNFα fails to induce cellular programs associated with a block of proliferation as seen in uninfected KCs (Chapter 5). The IFN pathways seems to be centrally attacked through downregulation of STAT1 levels which is observed in hrHPV episome-baring KCs when compared to uninfected KCs [14-16]. Downregulation of STAT1 results in attenuated ISG expression, albeit that signaling downstream of the IFNAR and IFNγR still functions (Chapter 5 and [15]). Thus, the attenuated type I IFN-induced ISG expression in HPV+ KCs must be due to the basal lowered STAT1 levels. In contrast, in experiments where E6 is overexpressed, E6 was shown to bind TYK2 and to interfere with STAT1 and STAT2 phosphorylation [17], implying that also STAT1 signaling is hampered by E6. If E6 plays a similar role in early infection remains to be determined. Importantly, IFNγ and TNFα stimulation induced processing of the non-canonical NFκB precursor p100 into p52 in hrHPV-infected cells but not uninfected KCs (Tummers, *Unpublished data*), indicating that hrHPV skews the response of KCs upon stimulation with TNFα and IFNγ towards the non-

canonical NFκB pathway. Potentially, this is caused by E7 as this oncoprotein was shown to increase SCF-βTrCP protein levels [18] and in this way might accelerate p100 processing [19]. Although unexplored at this point, it is highly likely that this forms another pathway allowing hrHPV-infected cells to resist control of infection by the immune system and the anti-proliferative effects of IFNγ and TNFα (Chapter 5).

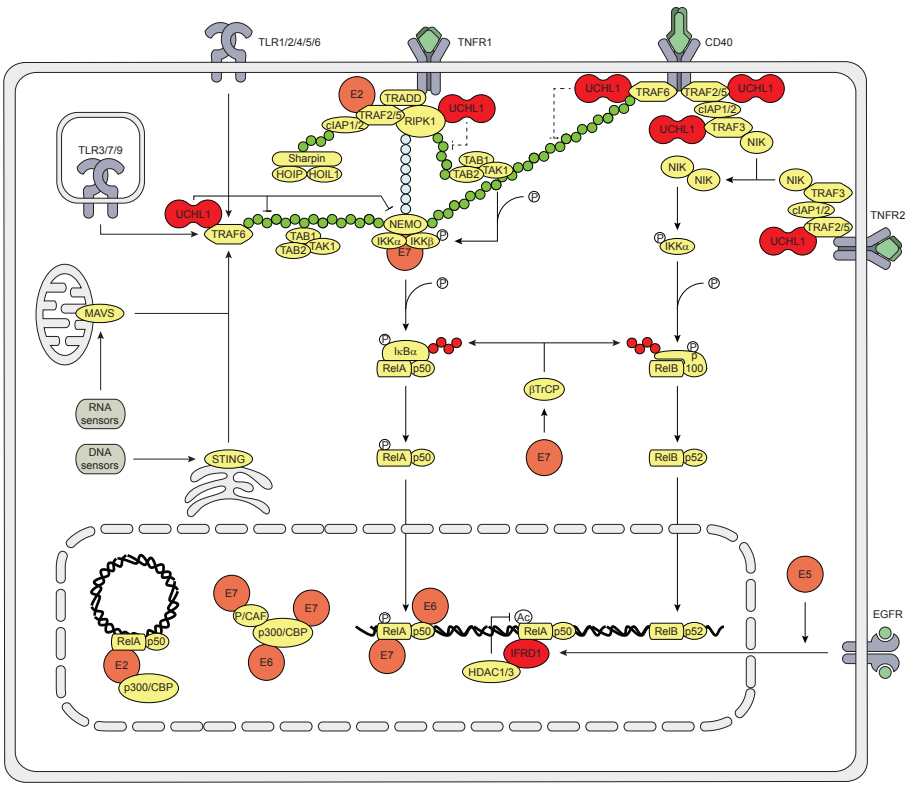

Figure 2: The effects of hrHPV on NFκB signaling

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 own encoded E proteins (red) as well as exploits the cellular proteins (red) UCHL1 and IFRD1 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. Dashed lines indicate hypothetical effects.

Epithelial cells express CD40 on their cell surface [20] and ligation of CD40 induces both canonical and non-canonical NFκB signaling, similar to TNFR1 and 2, respectively [21]. We showed that ligation of CD40 on epithelial cells results in a very coordinated response by KCs, dominated by the expression of genes involved in leukocyte migration, cell-to-cell signaling and interaction, as well as cell death and survival. The presence of HPV does not affect the gene expression profile of CD40 stimulated KCs, but it does attenuate the extent of the response and reduces the attraction of PBMCs (Chapter 3), indicating that the virus also attenuates CD40-induced signaling. Based on our previous studies it is likely that the CD40 – NFκB1 axis of CD40 signaling is affected via the interaction of UCHL1 and TRAF6, the effects of E7 on the IKK complex, and that of IFRD1 on NFκB1 transcriptional activation. Speculatively, at the non-canonical side signaling could be hampered by abrogation of UCHL1-mediated TRAF2 and/or 5- or E7-mediated IKKα functioning. However, UCHL1-mediated TRAF3 hampering could also lead to constitutive NIK accumulation and subsequent pathway activation (Figure 2). It remains to be determined if hrHPV prefers to skew KCs towards non-canonical NFκB activation after CD40 ligation.

NFκB signaling in hrHPV transformed cells

In contrast to hrHPV-infected cells, higher intraepithelial neoplastic lesions and HPV-positive cancers often show overactive canonical NFκB gene expression [22]. Indeed, overexpression experiments showed that E6 and/or E7 can also have pro-NFκB signaling effects and can increase NFκB target gene expression [16]. Mechanistically, E6 targets the NFκB repressor NFX1-91 for degradation [23] and under hypoxic conditions hampers CYLD, a negative regulator of NFκB signaling [24]. E6 also upregulates gene expression of the NFκB signaling components p50, NIK and TRAIP [16]. E7 upregulates SCF-βTrCP protein levels [18], which might lead to accelerated IκBα degradation

and p100 processing [19]. The transformed cell may benefit from E6/E7-enhanced NFκB signaling by maintaining a proliferative, anti-apoptotic state, although also pro-inflammatory cytokine expression is increased. Notably, cell type and growth rate are important determinants whether HPV E6 or E6/E7 stimulate or inhibit NFκB activation [25], and since viral gene expression considerably differs between hrHPV-infected KCs and hrHPV-transformed cells, data obtained from viral protein overexpression experiments should be carefully interpreted with respect to what their effects are in infection or cancer.

How HPV regulates cellular gene expression remains unclear

How HPV differentially expresses the genes studied in this thesis is still under investigation, but the episomal nature of the viral genome and it's translation into polycistronic mRNA make it difficult to study the functions of the individual E proteins in the context of a primary infection. We have overexpressed the individual early genes, their combinations and all combined in basal KCs, HaCat cells and primary fibroblasts, but, although the early genes were expressed, we could not detect differential expression of UCHL1, IFRD1 or IFITM1 in any of these overexpression experiments (Tummers, *Unpublished data*). Since plasmid-based overexpression of the early genes does not count for the effects of the episome itself, the presence of the viral episome in regulating cellular gene expression must be important. Transcription of the episome produces a polycistronic mRNA strand that completely disintegrates with current siRNA techniques directed at a single early gene. siRNA directed against E6, E7 (Tummers, *Unpublished data*) or E2 abolishes expression of the other early genes and abrogates the HPV-induced differential expression of UCHL1 (Chapter 2), IFRD1 (Chapter 4), and IFITM1 (Chapter 5), indicating that episome presence is indeed necessary in regulating cellular gene expression. Since single early genes cannot be knocked-down in our model, generating KCs harboring episomes with specific mutations in a gene, rendering the gene functionally inactive without influencing the other genes or polycistronic mRNA strand, could be a way to study specific early genes. This could give important insights into the function of an early gene in the context of early infection, but unfortunately, no such system exists to date.

Genetic predisposition to developing HPV-induced malignancies

Most HPV infections resolve spontaneously, although HPV invests heavily in suppressing host immunity. This indicates that external factors, such as genetic and environmental factors may contribute to the establishment of a persistent infection and progression to cancer. Genetic predisposition to cervical tumors was found [26] and several combinations of single nucleotide polymorphisms (SNPs) were associated with an increased risk to cancer. SNPs in genes of the antigen processing machinery, such as HLA-A, LMP7, TAP2 and ERAP1 [27], and in the FANCA and IRF3 genes [28] were linked to persistent HPV infection and formation of cancer. SNPs in the TLR and NFκB pathways were also studied [29]. Of the thirty-two candidate genes involved in these pathways, including TLR3, NFκB1, NFκB2, RelA, RelB, TRAF3 and TRAF6, only a SNP in the 5' UTR of the lymphotoxin alpha (LTA; TNF superfamily member 1) was significantly associated with increased risks of cervical and vulvar cancers [29]. Based on the interactions between the different proteins in the downstream signaling pathways and their outcomes with respect to activation, splicing, degradation and translocation it might well be that combinations of SNPs, of multiple genes associated with the IRF and NFκB pathways, rather than single SNPs, may confer protection or susceptibility towards persistence of HPV infection.

Final comment

Being a small virus, HPV relies on just 6 encoded early proteins, and some splice variants thereof, to interfere with normal KC physiology. Although the early proteins have a variety of cellular protein targets, it is remarkable that the virus only needs so few encoded genes to persist. Our work showed that HPV, via yet unknown ways, exploits cellular proteins to achieve its goals.

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