Human papillomavirus (HPV) 16 E6 oncoprotein targets the Toll-like receptor pathway

Lucas Boeno Oliveira,1,* Ismar R. Haga2 and Luisa Lina Villa3,4

Abstract
Cervical cancer is one of the leading causes of death in women worldwide and is etiologically linked to human papillomavirus (HPV) infection. Viral early proteins E6 and E7 manipulate cellular functions to promote the virus life cycle and are essential to the cellular transformation process. The innate immune system plays a pivotal role in the natural history of HPV infection. Among the various proteins that mediate the innate immune response, Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) and initiate the immune response. The objective of this study was to identify HPV E6 protein interaction partners in the TLR signalling pathway that may play a role in the immune response against HPV. Six TLR pathway proteins were shown to interact with HPV16 E6: myeloid differentiation primary response protein (MyD88), TIR domain-containing adapter molecule 1 (TRIF), interleukin-1 receptor-associated kinase-like (IRAK) 2, TNF receptor-associated factor (TRAF) 6, I-κB kinase beta (IKKβ) and I-κB kinase epsilon (IKKe). The interaction site of IKKe with E6 is located in the region containing the enzyme catalytic site, suggesting an influence of E6 on the activation of IKKe target proteins. HPV16 E6 potentiated the activation of NF-κB by various TLR pathway members. These results suggest that HPV16 has the ability to interfere with components of the immune response, contributing to HPV carcinogenesis.

INTRODUCTION
The innate immune system represents the first line of host defence against microbial invasion. It acts to prevent pathogen entry into the host, provide early detection and warning of their presence, regulate inflammation, activate the adaptive immune response, actively combat infection and maintain homeostasis. This response is often mediated by cell surface proteins such as Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs) [1]. These transmembrane receptors initiate a cascade of molecular events involving many intracellular proteins, culminating in the activation of transcription factors such as nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), IFN regulatory factor 3 (IRF-3) and IFN regulatory factor 7 (IRF-7), which, in turn, leads to the transcription of pro-inflammatory genes, T-cell stimulators, cytokines and antiviral response genes, including interferons. Lipopolysaccharides, flagellin, double-stranded RNA and non-methylated DNA are examples of PAMPs recognized by TLRs [2, 3].

Cervical cancer is a major cause of cancer mortality worldwide and the fourth most common type of cancer among women. It is estimated that every year 530 000 new cases are diagnosed worldwide and 270 000 women die from the disease, 86.6 % of whom live in less developed regions [4]. This type of cancer is etiologically linked to infection by certain types of human papillomavirus (HPV), which are epitheliotropic and species-specific DNA viruses. In addition to cervical cancer, HPV infection has also been linked to anal, vulvar, vaginal, penile and oropharyngeal cancer, and HPV16 is the most common of the HPV types found in HPV-related cancers [4].

The persistence of HPV is a key event in the natural history of HPV infection and cervical carcinoma development and is associated with viral ability to evade the host immune system [5]. Studies have shown that the adaptive response to HPV infection can take months to develop [6] and that components of the innate immune system may play a decisive role in the outcome of infection [7, 8].
During the course of infection, the viral genome can integrate into the genome of the human cell. Because HPV are double-stranded circular DNA viruses, breaks must be created in the circular viral episome for integration to occur. Breaks are frequently found in the HPV16 E2 gene region, a negative transcription regulator of the HPV early genes, and its absence leads to upregulation of E6 and E7 expression, which are major transforming HPV oncoproteins [9]. E6 plays an essential role in cell transformation and has the ability to interact with a wide variety of host proteins and, through these interactions, modify important cellular processes such as cell survival, gene transcription, cell differentiation, dependence on growth factors, response to DNA damage and cell cycle progression [10–12].

Keratinocytes are natural targets of HPV infection and can express different TLRs on their cell surface or inside endosomes [13, 14]. The expression of oncoproteins from high-risk HPV, which are capable of causing carcinomas, but not from HPV types with low carcinogenic potential, can inhibit TLR9 mRNA expression and protein activation in keratinocytes [15, 16]. Consistently, it was demonstrated that regression of HPV16 infections is significantly associated with increased expression of TLR2, TLR3, TLR7, TLR8 and TLR9 [17]. These findings suggest that modulation of the TLR-triggered immune response plays a protective role against HPV infections and can be decisive in regard to viral persistence or clearance.

Some proteins of the TLR signalling pathway have been recognized as potential therapeutic targets in cancer. The adaptor protein myeloid differentiation primary response protein (MyD88) has a pivotal role in the TLR response, being canonically the first step of the signalling pathway of all TLRs, except for TLR3 and TLR4 in endosomes [2]. Despite its importance in the immune response, MyD88 may have a pro-tumour role by participating in the inflammation process and thus contributing to carcinogenesis in the skin, liver, pancreas and colon [18–20]. Because the immune response and inflammation play a significant role in HPV carcinogenesis [21], it is possible that MyD88 is involved in the progression of HPV-related diseases.

In contrast, IκB kinase epsilon (IKKe) acts downstream of the adaptors in the TLR signalling pathway, and is part of the ‘MyD88-independent’ pathway. It can activate NF-κB and IRF-3, being essential to the antiviral response [22]. This kinase acts as an oncogene in breast cancer [23–25], coordinates invasion and metastasis in ovarian cancer [26] and stimulates proliferation and invasion in gliomas [27]. IKKe can also serve as a protective factor against DNA damage-induced cell death [28] and has become a potential therapeutic target for autoimmune diseases and certain cancers [29]. In addition, there is evidence that IKKe plays an important role in viral carcinogenesis [30, 31].

Several studies have investigated treatments aimed at enhancing the innate immune response to fight HPV infection [7] using agonists for TLR9 [32–34], TLR8 and TLR7 [35], or TLR4 and TLR3 [36]. Nevertheless, during the course of the infection, the host immune response can help promote tumour progression through inflammation processes [21, 37]. Accordingly, Tindle et al. [38] argued that HPV evades rather than completely inhibits the immune system.

To better understand the role of the innate immune response and Toll-like receptors on HPV infection and carcinogenesis, this study investigated the interaction of HPV with members of the TLRs signalling cascade using protein interaction and reporter gene assays.

**RESULTS**

**Interactions between HPV16 E6 and TLR pathway members**

To investigate the interaction between HPV16 E6 and proteins involved in the TLR pathway, we performed
co-immunoprecipitation assays. We observed that E6 interacts with MyD88 (Fig. 1a), TIR domain-containing adapter molecule 1 (TRIF) (Fig. 1b), interleukin-1 receptor-associated kinase-like (IRAK) 2 (Fig. 1c) and TNF receptor-associated factor (TRAF) 6 (Fig. 1d). However, no interaction was observed between E6 and IRAK4 (Fig. 1e).

The IkB-kinases involved in the TLR pathway were also analysed. IKKβ and IKKε were found to be E6 interaction targets, as opposed to IkB kinase alpha (IKKα) and TANK-binding kinase 1 (TBK1) (Fig. 1f). The specificity of the interactions observed between HPV16 E6 with IkB-kinases was remarkable. Thus, to determine the region of IKKε responsible for the interaction with HPV16 E6, we constructed six recombinant plasmids encoding FLAG-tagged truncated IKKε proteins. The analyses revealed that the interaction is mediated by the amino-terminal portion of the kinase, where the catalytic site of the enzyme is located (Fig. 2b, c). Remarkably, this interaction was observed in HeLa cells, a HPV-18 positive tumour epithelial cell line (Fig. 2a).

It is important to mention that samples containing E6 interaction partners displayed higher concentration of the E6 protein (Fig. 1), suggesting that the interaction could have been favoured by higher availability of the substrate. However, several studies have previously reported that the interaction of E6 with other proteins stabilizes the viral protein, thereby affecting E6 levels [39, 40]. This stabilization is even more evident in heterologous overexpression systems, resulting in E6-enriched protein extracts in solutions containing an interaction partner protein.

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**Fig. 2.** IKKε interaction with HPV16 E6 occurs in the amino-terminal portion of the protein. (a) Interaction study in HeLa cells transfected with plasmids coding for IKKε and/or HPV E6; HEK 293T cells were used as positive control. (b) Truncated IKKε protein constructs map. (c) HEK 293T cells were transfected with HA-tagged HPV16 E6-coding plasmid and plasmids coding for FLAG-tagged wild-type or truncated IKKε, as indicated in the figure. Wt: wild-type (whole protein); KD: kinase domain. After 24 h, lysates were subjected to immunoprecipitation, SDS-PAGE and immunoblotting with the indicated antibodies.
Because some of the identified E6 interaction partners belong to the same signalling pathway and are known to interact with each other, it is possible that the E6 interactions observed were indirect. In this case, indirect partners would have been immunoprecipitated as a complex when the direct partner was pooled down. Further experiments to distinguish between direct and indirect E6 interaction partners can contribute to understand the relationship of E6 and TLR signaling.

**NF-κB activation**

Because NF-κB is an important TLR response effector molecule, we studied the effect of HPV16 E6 on NF-κB activation upon transient expression of TLR pathway members. We observed that E6 significantly potentiated the activation of NF-κB by MyD88, TRAF6, MYD88–adaptor-like (MAL), IRAK2 and IKKε, whereas only slight increases were observed when TRIF-related adaptor molecule (TRAM), IRAK4 or TRIF were co-expressed with E6 (Fig. 3). However, E6 expression did not affect cells transfected with TBK1 or control cells (Fig. 3).

Activation of NF-κB by the proteins previously identified as E6 interaction targets was potentiated by the presence of the viral protein. Although IKKβ is also a known interactor, its influence on NF-κB activation was not investigated.

No increase in NF-κB activity was observed in cells transfected with E6 alone. Thus, in our system, HPV16 E6 does not enhance NF-κB activity per se, but rather in the presence of TLR pathway members, indicating that E6 acts through the TLR pathway to affect NF-κB activity. These results show that the interactions described above alter the biological activity of TLR pathway members, enhancing the immune response-related activation of NF-κB.

**MyD88 and IKKε expression**

Interactions with HPV E6 proteins can alter the concentrations of its target proteins (16). We therefore quantified the relative concentrations of MyD88 and IKKε proteins in the presence of HPV oncoproteins by Western blot. MyD88 and IKKε proteins were detected in primary human keratinocytes (PHK), C-33 A, HaCaT, SiHa, CaSki, HeLa and HTE cells.

Comparatively, C-33 A cells showed the lowest levels of both proteins, which were sometimes undetectable (Fig. 4). However, the presence of HPV E6 or E7 proteins was not sufficient to affect the endogenous concentration of MyD88 (Fig. 4a) or IKKε (Fig. 4b). This observation is even more evident when

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**Fig. 3.** HPV16 E6 interferes with with NF-κB activation mediated by TLR pathway members. HEK 293T cells were transfected with coding plasmids: 25 ng of MyD88, 50 ng of TRAF6, 25 ng of MAL, 50 ng of TRAM, 50 ng of IRAK2, 50 ng of IRAK4, 15 ng of TRIF, 50 ng of TBK1, 25 ng of IKKε and increasing amounts (25, 50 and 100 ng) of HPV16 E6. NF-κB luciferase reporter gene activity was measured 24 h after transfection. One representative experiment of at least three independent experiments is shown. * P-value <0.05.
we compare HTE cells expressing either HPV16 E6 or E7 or both proteins combined. HEK 293 and HEK 293T cell lines did not express MyD88 or IKKβ at detectable level (data not shown). These results demonstrate that, although MyD88 and IKKβ levels vary among the cell lines tested, there is no evident relationship with the presence of HPV oncoproteins.

**DISCUSSION**

The HPV E6 oncoprotein manipulates the host cell biology by interacting with and modulating the activity of various cellular proteins. In this study, we showed that the TLR response pathway is also an E6 target. HPV16 E6 protein was able to interact with several members of the TLR pathway and enhance NF-κB activation.

Two possibilities exist regarding the action of E6 on the immune system: response inhibition to escape the immune system for infection establishment, and response activation to eliminate the episomal form of the virus and promote tumour progression. Our findings suggest that the immune response mediated by TLR signalling is stimulated by HPV16 E6.
In the current study, we observed that E6 interacts with six proteins involved in the TLR signalling pathway: MyD88, TRIF, IRAK2, TRAF6, IKKβ and IKKe. Interestingly, because some of these proteins interact with each other in the TLR signalling cascade, we cannot rule out the possibility that the interaction of E6 with some of these occurs indirectly. Nevertheless, the large number of HPV16 E6 molecular partners in a signalling pathway responsible for immune response against invading microorganisms is staggering.

Notably, we observed that E6 interacts selectively with members of the IκB kinase family. In our model, E6 was able to interact with IKKe and IKKβ, but not IKKα and TBK1. This is interesting considering the high homology between these enzymes, especially in the enzymatic domain where interaction with IKKe was demonstrated. The primary structure of the TBK1 kinase domain shares 67% sequence identity with that of IKKe [29]. Considering the large number of known E6 interaction partners, there is much discussion about the relevance of the interactions [41]. Nevertheless, the observed specificity provides a strong indication for a biological basis to these interactions.

We observed that the interaction site of IKKe with HPV16 E6 is located within the first 160 amino acids of the enzyme. This finding is interesting because the catalytic site and ATP binding site of the kinase are located in this region [42]. Through this interaction, E6 can have a significant effect on the activity of NF-κB and IRFs, which are activated by IKKe. We observed that E6 activates NF-κB in conjunction with IKKe, but is inactive in the presence of, and does not interact with, TBK1. However, NF-κB activation was also enhanced by MyD88, MAL, TRIF, IRAK2 and TRAF6, which reinforces the hypothesis that HPV16 E6 plays an activating role in this segment of the immune response.

It has been widely reported that viruses can activate the immune system and promote disease progression [43–47], and this process can be mediated by proteins such as IKKe [48] and MyD88 [20]. The activation of NF-κB by HPV16 E6 had previously been described [49], and despite its role against infection, it has been shown that NF-κB is often essential for the initiation and progression of cancer [50]. Mine et al. [51] investigated the changes in gene expression in cervical cancer patients and observed a steady increase in the expression of genes related to antiviral response and cell cycle, demonstrating that these processes can be important for tumour progression.

Vandermark et al. [52] showed that HPV16 E6 and E7 proteins inhibited NF-κB activity in cultured cervical epithelial cells and suggested that this process contributes to malignant transformation. However, this effect was particularly related to the E7 protein and differed between cell cultures in different states of confluence and proliferation. For instance, HPV16 E6 increased NF-κB activity in non-confluent untreated cells [52]. In our study, all experiments were performed with proliferating cells; we did not use cells grown to a completely confluent state.

The influence of E6 on transcription factors is well known, and this viral protein has been shown to interact with IRF-3 and prevent transactivation of its target genes [53]. Activation of NF-κB and inhibition of IRF-3 has also been observed in herpes simplex (type 1), influenza and hepatitis C virus infections [54–56].

Many E6 actions depend on its ability to interact with multiple human proteins. Numerous interactions have been identified, but few experiments have been performed with keratinocytes, which are naturally infected by HPV. E6 protein interactions have been investigated using in vitro transcription and translation systems, CV-1 cells (monkey kidney fibroblasts), renal HEK 293/293T cells, Escherichia coli, NIH 3T3 cells (mouse fibroblasts) and gastric BGC823 cells, among others [11]. The low proliferative capacity, short lifespan ex vivo and reduced heterologous protein expression of keratinocytes have been an obstacle to observing protein interactions in these cells. In our study, we successfully demonstrated the interaction between E6 and IKKe in cells that are naturally infected with HPV. Although to a much lesser extent than that found in HEK 293T cells, this result is highly relevant for molecular target studies of E6 and shows, with high reliability, that this interaction can occur during the course of natural HPV infections. On the other hand, the co-immunoprecipitation assays were not able to detect interaction of endogenous proteins in any cell lines (data not shown), indicating that a more sensible method is needed to test the interactions without using heterologous expression.

The expression and activity of different TLR pathway members, from transmembrane receptors to transcription factors, can be affected by HPV16 E6 at multiple steps of the pathway (16, 58). Our findings suggest that the TLR pathway is important for the success of viral infection and/or disease progression. Future studies should investigate the biological significance of our findings and help identify new E6 targets. Despite the growing interest in exploring activation of the immune system as a treatment for HPV infection [57–59], we believe that such therapies should not be used before fully understanding the immune responses to papillomaviruses and before ruling out the possibility of tumour progression being facilitated by antiviral action.

**METHODS**

**Cell lines**

HEK 293T, CaSkii, SiHa, HeLa, C-33 A and HaCaT cells were acquired from ATCC (Manassas, VA, USA) and cultured as recommended by the manufacturer in culture media supplemented with 10% (v/v) fetal bovine serum (FBS). Primary human keratinocytes were acquired from Lonza (Basel, Switzerland) and grown in keratinocyte serum-free medium (KSF) supplemented with epidermal growth factor 1–53 and bovine pituitary extract (Thermo Fisher, Waltham, MA, USA). Human tonsil epithelial (HTE) cells were immortalized with stable transduction of the human telomerase gene and silencing of the tumour suppressor gene p16 [60], which were kindly provided by...
McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, USA. HTE cells stably carrying LXSN vector alone or engineered to encode HPV16 E6, E7 or both E6 and E7 were cultured in KSFM (with supplements). All cells were cultured at 37 °C in a humidified atmosphere of 5 % CO₂.

**Plasmids and molecular cloning**

The plasmid coding for HPV16 E6 (HA-tagged) was kindly donated by the International Centre for Genetic Engineering and Biotechnology (Trieste, Italy). Plasmids coding for IKKα and IKKβ (FLAG-tagged) were donated by McArdle Laboratory for Cancer Research (Madison, WI, USA). The remaining plasmids used (MyD88-AU1, IRAK2-Myc, FLAG-TRAF6, FLAG-IRAK4, TRAM, FLAG-TRIF, FLAG-IKKα and FLAG-TBK1) were kindly donated by Trinity College Dublin (Ireland) and the University of Massachussetts (Worcester, MA, USA).

Plasmids encoding truncated IKKα proteins were generated by PCR from the plasmid containing the complete coding region of IKKα [22]. The amplicons were inserted into the pcDNA3 vector between BamHI and XbaI restriction sites. The truncated IKKα proteins are composed of amino acids 1–160, 1–315 (kinase domain of the protein), 1–569, 1–717 (native protein), 315–569 and 315–717. Six oligonucleotides, two forward primers (5′ agtcagcatctcagcaacagcagcacaattac 3′ and 5′ agtcatagttctggttcagtgagcc 3′) and four different reverse primers (5′ atgccatgtcaagacgccgaaagtcctgac 3′, 5′ aggcatctactagcagacatgacgacaaac 3′, 5′ atgtcaactacatgatgacgagccggtgctg 3′ and 5′ tgcagttctagcatcagcatcagagtgctg 3′) were used.

**Cell transfection and protein extraction**

HEK 293T cells were cultured in 100 mm-diameter dishes in DMEM supplemented with 10 % FBS. The transient transfections were performed using 4 µg of each plasmid and FuGENE6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer’s instructions at a 3 : 1 ratio. After 24 h, cells were washed with PBS buffer and harvested with 500 µL of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 % TRITON X-100) containing complete Protease Inhibitor Cocktail (Merck, Darmstadt, Germany) [16]. The suspension was incubated on ice for 20 min with mild agitation and centrifuged at 16 000 × g for 15 min at 4 °C to obtain the purified protein supernatant. A similar procedure was used for HeLa cells, but transfections were performed using 6 µg of each plasmid and FuGENE HD (Promega) transfection reagent.

**Immunosassays**

FLAG-tagged proteins were precipitated with anti-FLAG M2 affinity gel (Sigma–Aldrich, St. Louis, MO, USA) following the manufacturer’s protocol. For all other immunoprecipitations, the appropriate antibodies were pre-coupled to either protein A- or G-Sepharose overnight at 4 °C before incubation with the cell lysates. Cell lysates were incubated with the antibodies overnight at 4 °C with constant agitation. The immune complexes were precipitated by brief centrifugation at 7 000 g, washed twice with ice-cold TBS buffer and eluted with SDS sample buffer. Eluted extracts and crude protein solutions (input) were analysed by SDS–PAGE and immunoblotting.

Western blot assays were performed using 0.1 % TBS–TWEEN, pH 7.4 buffer for antibody dilutions and in the blocking and washing steps. The primary antibodies used were: anti-AU1 (BioLegend, San Diego, CA, USA), anti-FLAG (Sigma–Aldrich), anti-HPA (Sigma–Aldrich), anti-IKKα (Abcam, Cambridge, UK), anti-iMyD88 (Abcam), anti-tubulin (Sigma–Aldrich) and anti-HPV16 E6 (Arbor Vita, Freemont, CA, USA).

The membranes were analysed by chemiluminescence on an ImageQuant LAS imaging system (GE Healthcare, Chicago, IL, USA). The signals were quantified using ImageQuant TL software (GE Healthcare). For cell expression analysis, up to seven experiments were conducted to calculate mean expression values normalized to tubulin expression. Mean values were compared by two-tailed paired t tests.

**Reporter gene assays**

HEK 293T cells were seeded into 96-well plates (2 × 10⁴ cells per well) and transfected 24 h later with the expression vectors and 60 ng/well of a κB-luciferase gene reporter plasmid using GeneJuice (Merck). In all cases, 20 ng/well of pGL3-Renilla was co-transfected to normalize data for transfection efficiency. The total amount of DNA per transfection was maintained at 230 ng by the addition of pcDNA3.1 empty vector. After 24 h, reporter gene activity was measured. Data are expressed as the mean fold induction± SD relative to control levels for a representative experiment from at least three independent experiments, each performed in triplicate. Means were compared by two-tailed unpaired t tests.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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