The handle http://hdl.handle.net/1887/32931 holds various files of this Leiden University dissertation

Author: Karim, Rezaul  
Title: Deregulation of innate and adaptive immune responses in human papillomavirus infection and cancer  
Issue Date: 2015-05-07
Human Papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes

Chapter 2

Rezaul Karim, Craig Meyers, Claude Backendorf, Kristina Ludigs, Rienk Offringa, Gert-Jan B. van Ommen, Cornelis J. M. Melief, Sjoerd H. van der Burg, Judith M. Boer

PLoS One. 2011 Mar 14;6(3):e17848.
Human Papillomavirus Deregulates the Response of a Cellular Network Comprising of Chemotactic and Proinflammatory Genes

Rezaul Karim1,2,3, Craig Meyers4, Claude Backendorf5, Kristina Ludigs22a, Rienk Offringa22b, Gert-Jan B. van Ommen1, Cornelis J. M. Melief5, Sjoerd H. van der Burg5, Judith M. Boer1,6a

1 Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands, 2 Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands, 3 Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands, 4 Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania, United States of America, 5 Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands, 6 Netherlands Bioinformatics Centre, Nijmegen, The Netherlands

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 papillomaviruses (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 HPV. Many of the genes downregulated in HPV-positive KCs involved components of the antigen presenting pathway, the inflammassome, 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.

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 OutoChip 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-inhibitor 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 observable.

We aimed at understanding the effects of high-risk HPV infections 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-1 pathway [30–32]. Here, we show that HPV could be a common 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 IL1R.

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 γ-irradiated mouse ST3 fibroblasts. Passage 4–5 of primary KCs - devoid of contaminating cells - were grown in serum-free medium (Defined KSF, Invitrogen, Breda, The Netherlands). Partial differentiation was induced by 1.8 mM Ga2+ for 24 hrs, terminal differentiation by placing KCs in single-cell suspension into serum-free medium containing 1.75% methylcellulose and 1.8 mM Ga2+ for 24 hrs [35]. 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 grew 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 ST3 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 mycoplasma. Where indicated, cells were stimulated with poly(LC) (25 μg/ml, InvivoGen, San Diego, USA), CCL2 and IL-1β 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, 1:900). 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). Real-time 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 SPRs [35]. Pre-designed primers and probe mixes for TLR3, CCL5, IL1B, RNASE7, NLRP2, and G4PDH 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 G4PDH 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 ES62 (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(LC). Total RNA for these 24 samples was isolated as stated above, and analyzed on an RNA 6000 Nano Lab-chip using 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, Streeterville, 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
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 v19.1.577 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] hasH620 (7/17/09) with improved layout using PathVisio v1.0 beta software [44]. The edited pathway is available from GMAPP 2.1.0.5 and WikiPathways [45].

We used CORE_R [www.lrg.nl/CORE_R] 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 (100 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.

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 showed essentially the same pattern of PRR expression as Ramos B-cells and monocytes (Fig. S1B). The expression in KCs is consistent with work by others showing that TLR4 was only found in HaCat cells, but not in primary human KCs [16,48]. The absence of TLR4 expression in differentiated KCs, which was confirmed by immunohistochemistry in human foreskin and cervical epithelia (Fig. S2), 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 TLR4 expression in differentiated HPV-positive KCs was similar to that in HPV-negative cells. Thus, HPV did not affect mRNA expression of the tested PRRs.
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 (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) [15]. 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 654 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, TRAM1/TRIP, TRAM2/TRAM3) 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) showed upregulation of the Jak-STAT signaling pathway, triggered by temporary upregulation of IFNB1 after 4 hrs poly(I:C) through the TRAF5/TRKB signal transduction route, resulting in upregulation of STAT1 and chemokarzinik cytoxines CXCL12 and CXCL10. In summary, via TRAF6 the NF-kappaB 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 TRAM1 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 into 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), involved in differentiation and KC differentiation, fitting with the biological effect of HPV in delaying differentiation [50]. Cluster 2 genes (194 probes), including antimicrobials (DEFB105B, LO2728154, AQP9, RNASE7, SRC16), antigen presenting molecules (HLA-A, -B, -C, -G, HCP5), pro-inflammatory cytokines and chemokines (CCL5/RANTES, CCL2/GM-CSF, TGFP-alpha, IL7), interferon-inducible genes (IFIT2, IFIT4, TICAM1), and TRAM1 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), TRAM1 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 TRAM1 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 STAT3 (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
hrHPVs Suppress Immune Response in Keratinocytes

A

B

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, RARESS, CXCL16) that were stronger upregulated in HPV-positive than in
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(LC) 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(LC) 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-negative cells. We studied IL1B in more detail, since it represented a central target for HPV-mediated suppression of both 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 PTGER4/ASC, but not NLRP3, possibly contributing to the observed lower level of IL-1β. The most interconnected upregulated gene of the network was CDK2, 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 PRR 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 altered...
Chapter 2

hrHPVs Suppress Immune Response in Keratinocytes

PLoS ONE | www.plos one.org 8 March 2011 | Volume 6 | Issue 3 | e17848
hrHPVs Suppress Immune Response in Keratinocytes

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 inhibit 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 IL-6 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 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

Figure 6. HPVs downregulate IL1B and inflammasome components. (A), TaqMan RT-PCR showing pro-IL1β mRNA expression in control (HFK1 and HFK2) and HPV-positive (HV16 and HV18) KCs. (B), IL-1β protein secretion of control (HFK1 and HFK2) and HPV-positive (HPV16, HCK18 and HV16) KCs as measured by ELISA. (C), TaqMan RT-PCR showing NLRP2 mRNA expression in HPV-negative (HFK1, HFK2, HFK2) and HPV-positive (HPV16, HCK18, HV16 and HV18) KCs. In all three panels, data are mean ± SD, n = 3.

doi:10.1371/journal.pone.0017848.g006
suggesting that HPVs affect the TBK1 and NF-kappa-B signaling
TRAF3, TRAF6 and RIP1 [59]. Notably, the other poly(I:C)
regulatory factor (IRF) activation via downstream molecules
TLR3 pathway that mediates NF-kappa-B and interferon-
Additionally, it would be of interest to study if other viruses
into the molecular mechanisms involved in HPV-downregulated
differentiation genes.
closely mimicking the situation
differentiation and interaction with (innate) immune cells thereby
to perform a genome-wide study of HPV-positive KCs during
HPV-transformed cells. The strength of our study lies in the use of
lower secretion of IL-1
downregulated inflammasome components – needed to convert
fact that it may take months or even a year to control HPV
and T-cell immunity against early antigens E2 and E6. Cancer Res
10. de Vos van Steenwijk P, Piersma SJ, Welters MJ, van der Hulst JM, Fleuren G,
differentiated (3) normal foreskin keratinocytes. SPRR2A expres-
cycles in undifferentiated (1), partially differentiated (2) and fully
haematoxylin. Original magnification 125
normalized against GAPDH mRNA levels. Data represent an
a ''G''. (B), TaqMan real-time PCR was performed for TLR9 on
total RNA samples from indicated cell types. TLR9 expression was
a TLR9-specific or isotype control antibody of paraffin-embedded
oncoproteins, which is more relevant for our understanding of
studies relied on the overexpression of either one or both

We thank Enno Droef, Yusuf Ariyurek, and the Leiden Genome
Technology Center for excellent experimental assistance. We thank
Thomas Kelder and Martijn van Jersel for automatically extracted KEGG
pathways and GO terms and PathVisio beta software.

Acknowledgments

We thank Enno Droef, Yusuf Ariyurek, and the Leiden Genome
Technology Center for excellent experimental assistance. We thank
Thomas Kelder and Martijn van Jersel for automatically extracted KEGG
pathways and GO terms and PathVisio beta software.

Author Contributions

Conceived and designed the experiments: SHvdB CJMM GJ-B-O 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 GJ-B-O CM CB KL.
References

1. Frey, J., Shiue, H.R., Bray, F., Forman, D., Maders, C., et al. (2010) Estimates of worldwide burden of cancer in 2008. GLOBOCAN 2008. Int J Cancer. 127: 2011-2017.

2. Preusker, R. (2007) Papillomaviruses in the carcinogenesis of human cancer - a brief historical account. Virchows Arch 451: 45-51.

3. Bos, F.C., Munro, M.M., Munro, N., Sherman, M., Janssen, A.M., et al. (1999) Perinatal transmission of human papillomavirus: a worldwide prospective. International biological study on cervical cancer (IBSCC). Study Group. J Natl Cancer Inst 91: 796-802.

4. Richarz, D., Kalisch, G., Toller P., Voehr, H., Abrahamsen, M.W., et al. (2007) The natural history of type-specific human papillomavirus infections in female university students. Cancer Epidemiol Biomarkers Prev 16: 405-409.

5. Tindel RN (2002) Immune evasion in human papillomavirus-associated cervical cancer. Nat Rev Cancer 2: 59-65.

6. Stanley M. (2004) Immune responses to human papillomavirus. Vaccine 24 Suppl 1: S16-S22.

7. Wei, Y.L., Sterling, J., Damay, I., Coleman, N., Crawford, R., et al. (2000) Characterizing the local immune responses in cervical intraepithelial neoplasia: a cross-sectional and longitudinal analysis. BJOG 107: 1616-1621.

8. van de Pol, M., van der Sten, C., van Berendonk, M., Keppmans- Antonissen, C., et al. (2005) Detection of human papillomavirus (HPV) 16-specific CD4+ T-cell immunity in patients with persistent HPV16-induced vulvar intraepithelial neoplasia: a clinical impact of unstimulated treatment. Clin Cancer Res 11: 5273-5280.

9. de Jong, A., van de Pol, M., van der Heijden, J.M., Doehn, J.W., Freun, G.J., et al. (2008) Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Res 68: 1082-1088.

10. de Vos van Steenwijk P., Persma SJ, Wellers MJ, van der Heijden J.M, Freun G.J., et al. (2008) Surgery followed by persistence of high-grade squamous intraepithelial lesions and cervical carcinomas in young females. Int J Cancer 122: 1405-1410.

11. Haas, U.A., Bates, E., Takeshita, F., Biliato A., Accardi R., et al. (2007) TLR9 activation induces interferon response gene expression and viral infection. J Exp Med 199: 1641-1650.

12. Chan, Y.E., Laeimins LA (2000) Micro array analysis identifies interferon-gamma- and virus-inducible genes in cervical epithelial cells. J Immunol 181: 2694-2704.

13. Kalali BN, Kollisch G, Mages J, Muller T, Bauer S, et al. (2008) Double-stranded RNA and signalling molecules in HPV-infected and uninfected keratinocytes. Table S1

14. Mempel M, Voelcker V, Kollisch G, Plank C, Rad R, et al. (2003) Toll-like receptor 9 (TLR9) activation induces interferon response genes and up-regulates proliferation-associated and NF-kappaB-targeted gene expression in human cervical cancer precursors. J Immunol 170: 1381-1386.

15. Lebre MR, van der Aa AM, van Bassens L, van Capel TM, Schumakers MJ, et al. (2007) Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. J Invest Dermatol 127: 331-341.

16. Kalisch G, Kalab CN, Veeckle V, Wallrich, B, Behrendt H, et al. (2005) Various members of the Toll-like receptor family contribute to the innate immune response of human epithelial keratinocytes. Immunology 114: 331-341.

17. Andersen JM, Akiyoshi H, Ingram RR (2006) Innate immunity at the mucosal surface: role of toll-like receptor 3 and toll-like receptor 9 in cervical epithelial cells against viral infections. J Exp Med 199: 1641-1650.

18. Hemmi H, Takeuchi O, Sato S, Yamamoto M, Kato T, et al. (2004) The roles of two IlkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA-induced NF-kappaB activation. Cell 118: 1057-1064.

19. Schuitemaker EC, van der Heijden J, Freun GJ, et al. (2000) E2b, a putative nuclear export signal for human papillomavirus (HPV) E2. J Virol 74: 14774-14783.

20. Lemaire PA, Lavy JC (2005) Mechanism of PKR activation: dimerization and kinase activation in the absence of double-stranded RNA. J Biol Chem 330: 81-88.

21. Pehlman A, Schulte O, Tan CP, Nadzid T, Lijeski P, et al. (2006) RIG-I-family mediated antiviral responses to single-stranded RNA binding 4-9-180 domains. Science 314: 997-1001.

22. Sankar S, Chan H, Rommeijer WJ, Li J, Bates RJ, et al. (2000) BKV signals through BRF1 and NKBRI to mediate the production of inflammatory cytokines. Cell Signal 12: 988-993.

23. Yonesuma M, Kikuchi M, Matsumoto K, Utsunomiya H, Miyabayashi M, et al. (2003) Shared and unique functions of the DEAD/HEAT box helicases RIG-1, MDA5, and LGP2 in antiviral innate immunity. J Immunol 173: 2813-2818.

24. Jellum T, Hervig O, Kjoen J, Kyrre B, et al. (2007) The role of interferon regulatory factor-5 in interferon gamma-mediated antiviral defence. Biochim Biophys Acta 1779: 105-112.

25. Saito T, Takahashi K, Saito S, Caban C, Kuma H, et al. (2005) BPI-1, an adapter triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 6: 981-988.

26. Woodford DH, Cheng S, Sampson S, Hamacher L, Chow LT, et al. (1997) Reconstituent reoviruses containing human papillomavirus type 16 E6 and E7 genes stimulate proliferation and delay differentiation of human keratinocytes early after infection. Oncogene 6: 619-626.

27. Hafner J, Schroder JM (2002) Neat, a novel innate immune defense antimicrobial peptide of healthy human skin. J Biol Chem 277: 46779-46784.

28. Zhang J, Dye KD, Rosenberg HF (2003) Human Neat7, a new cationic ribosome-binding of the Neat A superfamily. Nature Immunol 3: 602-607.

29. Cabanne SE, Xiao W, Rachbach DB, Felciano RM, Baker HV, et al. (2005) A network-based analysis of system immunization in humans. Nature 437: 1032-1037.

30. Ghirlando F, Apetoh L, Ton armier A, Aymone L, Ma Y, et al. (2009) Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nat Med 15: 1170-1178.

31. Becker CE, O’Neill LA (2007) Inflammations in inflammatory disorders: the role of TLRs and their interactions with NLRs. Semin Immunopathol 29: 219-248.

32. Woss WL, van den Hende M, Sterling JC, Coleman N, Crawford RA, et al. (2019) A prospective study on the natural course of low-grade squamous intraepithelial lesions and the presence of HPV16E2, E6, and E7-specific T-cell responses. Int J Cancer 126: 131-141.

33. Wilte MS, van de Pol, M., van der Heijden, J.M., Doehn, J.W., Freun, G.J., et al. (2008) Detection of human papillomavirus type 16 E6 in cervical lesions is associated with interferon-regulatory factor-1 activity. Gene 422: 2061-2067.

34. Nevo M, Gesellschaften GM, Hyman T, Frank S, Miller L, et al. (2001) Papillomavirus type 16 oncoproteins downregulate expression of interferon-activated genes and upregulate proliferation and NP-kappaB-responsive genes in cervical keratinocytes. J Virol 75: 4281-4296.

35. Mertens LA (2000) Microarray analysis identifies interferon-inducible genes and Stat-1 as major transcriptional targets of human papillomavirus type 31. J Virol 74: 4174-4182.

36. Mertens LA, Mertens MG, Chmielewski SD, Meyers C. (2004) Ppoxylation, infection, and neutralization of authentic HPV16-virus. Virology 322: 213-219.

37. Meyers C, Moyer T, Usama MA (1997) Synthesis of infectious human papillomavirus type 18 in a transcriptionally competent cell line with viral DNA. J Virol 71: 7319-7326.
61. Huang SM, McCance DJ (2002) Down regulation of the interleukin-2 promoter by human papillomavirus type 16 E6 and E7 through effects on CREB binding protein/p300 and P/CAF. J Virol 76: 8716–8722.

62. Spitkovsky D, Helmer SP, Holtman TG, Moller A, Schmitz ML (2002) The human papillomavirus oncoprotein E7 attenuates NF-kappa B activation by targeting the I kappa B kinase complex. J Biol Chem 277: 25376–25382.

63. Barnard P, McMillan NA (1999) The human papillomavirus E7 oncoprotein abrogates signaling mediated by interferon-alpha. Virology 259: 805–813.

64. An J, Mo D, Liu H, Verma MS, Srinivasan ES, et al. (2008) Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-kappaB activation. Cancer Cell 14: 494–507.
