Mechanisms underlying the resistance of human papillomavirus-infected or -transformed cells to Th1 immunity

Ma, W.

Mechanisms underlying the resistance of Human papillomavirus-infected or -transformed cells to Th1 immunity

Wenbo Ma

Mechanisms underlying the resistance of Human papillomavirus-infected or -transformed cells to Th1 immunity

Author: Wenbo Ma

Cover lay out: Idea is provide by Wenbo Ma, cover is desighed by Remco Wetzels. Remcowetzels.nl

Thesis printing: Ridderprint BV, The Netherlands www.ridderprint.nl

ISBN number: 978-94-6375-150-6

Mechanisms underlying the resistance of Human papillomavirus-infected or -transformed cells to Th1 immunity

PROEFSCHRIFT

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. Mr. C.J.J.M. Stolker,

volgens besluit van het College voor Promoties

te verdedigen op dinsdag 18 december 2018

klokke 15:00 uur

door

Wenbo Ma

geboren te Lanzhou, China

in 1986

Promotoren:

Prof. Dr. S.H. van der Burg

Dr. T. van Hall

Promotiecommissie:

Prof. Dr. C.J.J.M. Stolker	
Prof. Dr. P. ten Dijke	LUMC
Mw. Dr. R.D.M. Steenbergen	VUMC, Amsterdam
Prof. Dr. E. Wiertz	UMC, Utrecht
Mw. Prof. Dr. M. Jager	LUMC
Prof. Dr. P.P.G. van Benthem	LUMC

The studies presented in this thesis were performed at the Department of Medical Oncology, Leiden University Medical Center, Leiden, The Netherlands. Wenbo Ma was financed by the Chinese Scholarship Council (CSC). The publication of this thesis was financially supported by the Leiden University Medical Center.

Table of contents

Chapter 1

Introduction

Introduction

1. Human papillomavirus and cancer formation
2. The immune response to HPV

    2.1 Innate immune response
    2.2 Adaptive immunity
    2.3 IFNγ signal transduction pathway and downstream effects
    2.4 TNFα signal transduction pathway and downstream effects
    2.5 Human papillomavirus-specific T-cell immunity

3. EGFR signaling
4. Scope of this thesis

1. Human papillomavirus and cancer formation

Human papillomaviruses (HPVs) are known to cause benign papillomas and some can cause epithelial malignancies. Human papillomavirus is a small non-enveloped virus with a diameter of approximately 55 nm. It is a double-stranded DNA virus with a genome of 8 kb in size [1]. The genome of HPVs contains three regions: early, late and long control regions (LCR). The early region contains open reading frames (ORFs) that encode the regulatory proteins E1 to E8. The late gene produces the structural proteins L1 and L2 that assemble into the viral capsid structure and are necessary for virion formation, transmission and spread [2].

Human papillomavirus infects keratinocytes (KCs) in the basal layer of the epidermis and mucosal epithelium, and its life cycle is linked to differentiation of infected KCs. The early proteins E1 and E2 regulate viral genome replication. High levels of E2 negatively regulate the expression of E6 and E7, allowing the cell to differentiate and enter the differentiation-dependent stage of the HPV life cycle. High levels of the viral protein E4 facilitate the release of progeny virions. The HPV proteins E6 and E7 stimulate cell growth, which allows the replication of the viral DNA, as well as increased viral early-gene expression. The late capsid proteins L1 and L2 are produced at the uppermost layer of the epithelium and assemble progeny virions, and finally new infectious viruses are released [3].

Human papillomaviruses are distinguished by their potential to cause malignant progression. Low-risk HPVs include HPVs 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81, which can cause low-grade lesions such as condylomas and genital warts. High-risk HPVs (hrHPV), including HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 can give rise to cancer [4] and account for approximately 5% of all cancers worldwide [5]. In the United States, hrHPV types are the cause of approximately 3% of all cancer cases among women and 2% of all cancer cases among men [6]. Virtually all cases of cervical cancer are caused by hrHPV, of which 70% are caused by only two HPV types: 16 and 18 [7-

9]. About 95% of anal cancers are caused by HPV, mostly type 16. About 70% of oropharyngeal cancers are caused by HPV and in the United States more than half of all cancers diagnosed in the oropharynx are linked to HPV type 16 [10]. In the Netherlands, the incidence of oropharyngeal squamous cell carcinoma related to HPV infection increased in the past decades (1980–2009) [11]. Human papillomavirus causes about 65% of vaginal cancers, 50% of vulvar cancers and 35% of penile cancers, most of which are caused by HPV type 16 [12]. Malignant cells retain the expression of the viral genes E6 and E7. The former binds to the p53 tumor suppressor protein and induces the degradation of p53, while the latter binds and inactivates the tumor suppressor retinoblastoma gene product (pRb). As a result, E6 and E7 deregulate the cell cycle, preventing apoptosis and differentiation, which lead to malignant transformation.

2. The immune response to HPV

HPV infection in most people is asymptomatic and the virus is cleared within two years. The host immune system plays an important role in the progression or regression of HPV infection. In some cases, HPV infections persist for an extended time, which indicates that HPVs have developed multiple mechanisms to escape immune-surveillance or resist the effector mechanisms of the immune system.

2.1 Innate immune response

The first defenses are the physical barriers provided by the skin and mucus, which prevent pathogens from entering the body. Any pathogen that succeeds in penetrating an epithelial surface immediately activates effector cells and molecules of the innate immune response. Innate immunity, also known as non-specific immunity, is an important protective system that reacts immediately when a host is invaded by pathogens.

Microbes express pathogen-associated molecular patterns (PAMPs) which can be recognized by the host's pattern-recognition receptors (PRRs), which are classified into two main classes: 1) membrane-bound receptors, including Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and 2) cytoplasmic sensors, including TLRs, NOD-like receptors (NLRs), pyrin and HIN domain containing (PYHIN) family members, RIG-like receptors (RLRs) and a range of cytosolic nucleic acid sensors [13]. Activation of these receptors leads to activation of the NF-κB pathway and/or type I IFN pathway to induce the production of immune-regulatory cytokines that dampen pathogen propagation.

Basal KCs express PRRs, including NLRs, TLRs and RNA helicases, to recognize PAMPs of microbes and viruses. In particular, KCs express TLRs 1–6 and 9 [14-16]. Activation of TLR2 on KCs by S. aureus results in activation of NF-κB, which subsequently increases the production of the neutrophil chemotactic factor IL-8 and iNOS [17]. In recognizing viral infections, TLR3 plays an important role. Keratinocytes activated via TLR3 may play an important role in antiviral immune responses in the skin. Activation of TLR3 by its ligand dsRNA (poly I:C) on human KCs induces the production of IL-8, TNFα, IL-18 and type I interferon (IFNα/β), as well as the development of Th-1 type immune responses [18-21]. Activation of TLR5 on KCs by its ligand flagellin results in production of TNFα, IL-8 and the antimicrobial peptides human β-defensins 2 and 3 (hBD2 and hBD3) [15, 21, 22]. Activation of KCs via TLR3 by poly I:C and via TLR9 by CpG DNA leads to selective production of chemokines CXCL9 and CXCL10, which promote the attraction of T cells and lead to the production of type I interferon [15].

Pathogens try to evade this immune-mediated detection. An infection with hrHPV can impair IRF3-mediated type I IFN signaling by downregulating STING and upregulating UCHL1, both of which results in the inactivation of TRAF3, with as a consequence a reduced TBK1-TRAF3 interaction and IRF3 phosphorylation [23, 24]. In addition, HPV38 E6 and E7 have been shown to inhibit the TLR9 promoter by

increasing ΔNp73α, which causes cell cycle deregulation [25]. Moreover, a reduced expression of TLRs 7 and 9 was found in the HPV-infected cervical epithelial cells of patients with systemic lupus erythematosus, suggesting that HPV downregulates their expression [26]. Similarly, the retrovirus-mediated overexpression of HPV16 E6 and E7 in KCs has been shown to downregulate TLR9, potentially forming an important strategy for its escape from immunosurveillance [27]. In HPV16-infected KCs, however, TLR9 was not found in the basal KCs, which are the target cells for HPV, but only in differentiated KCs. Hence, TLR9 downregulation may form a problem in the more differentiated cells, or the apparent loss of TLR9 is due to these cells' failure to differentiate [28]. Genome-wide expression profiles of control and HPV-positive undifferentiated (basal) KCs show that HPVs dampen TLR3, PKR, MDA5 and retinoic acid-inducible gene I (RIG-I) signaling. Hence, the innate immune response of KCs, which is normally able to alert innate and adaptive immunity, is impaired by HPV16 [28].

Retinoic acid-inducible gene I (RIG-I) is a key pattern-recognition receptor which recognize viral RNA and interacts with the mitochondrial adaptor MAVS.RIG-I triggers a signaling cascade which results in the type I interferons (IFNs) production. Human papillomavirus' E6 oncoprotein impairs the activation of the cytoplasmic innate immune sensor RIG-I by targeting TRIM25 and USP15 which are the upstream regulatory enzymes to hamper the innate immune response [29]. Innate-immunity-associated genes were analyzed by microarrays in human primary KCs transduced by HPV16 E2. E2 proteins of high-risk HPV decrease type I interferon IFN-κ and STING expression as well as the downstream target genes, which may be an immune-evasion mechanism involved in HPV persistent infection and cervical cancer development [30]. Interleukin-1 beta (IL-1β) mediates inflammation and also links innate and adaptive immunity. HPV16 E6 oncoprotein abrogates IL-1β processing and secretion, and IL-1β expression gradually decreases from HPV-positive tissue samples towards cervical cancer.

Attenuation of IL-1β by the HPV16 E6 oncoprotein is crucial in viral immune evasion and initiation of malignancy [31].

In addition, HPV may impair intracellular signaling pathways to allow virus-infected cells to resist the effector molecules of innate and adaptive immune cells. For instance, hrHPV interferes with IFNAR signaling, while HPV18 E6 binds to TYK2 to impair the phosphorylation of STAT1 and STAT2 [32]. Furthermore, E6 and E7 of HPVs 16 and 31 impair STAT1 transcription and translation, as well as the binding of STAT1 to ISRE [33]. STAT1 is the key component of IFNR signaling. Phosphorylated STAT1 is translocated into the nucleus and induces the expression of genes that have antiviral and antiproliferative effects. As a result, the suppression of STAT1 activity may impair the antiviral and antiproliferative activities of IFNAR1 signaling.

In addition, NF-κB plays a key role in regulating the immune response to infection. hrHPV has been shown to hamper NF-κB by upregulating the endogenous protein UCHL1, which results in NEMO degradation [24]. Furthermore, hrHPV induces epidermal growth factor receptor （EGFR）overexpression, increasing the expression of the cellular protein interferon-related developmental regulator 1 (IFRD1), which mediates RelA K310 deacetylation and thereby attenuates the transcriptional activity of NF-κB [34]. Finally, hrHPV infection can also attenuate CD40-induced NF-κB activation, resulting in impaired production of chemokines and the attraction of lymphocytes and myeloid cells following CD40 stimulation. As such, hrHPV impairs the local development and sustainment of adaptive immune responses [35].

2.2 Adaptive immunity

The adaptive immune response, also known as specific immune response, is the host's third line of defense. Adaptive immunity plays important role only after physical barriers are breached and the

innate immune response fails to clear the invading pathogens. Two types of lymphocytes are essential for the adaptive immune response: B lymphocytes and T lymphocytes. Unlike the innate immune response, the adaptive immune response is highly specific to a particular pathogen and provides long-lasting protection.

The adaptive immune response includes both humoral immunity mediated by B cells and cell-mediated immunity mediated by T cells. Humoral immunity can be classified according to the different types of antigens. Thymus-independent antigens, comprising bacterial products such as LPS, capsular polysaccharide and polymerized flagellin can directly stimulate B cells to induce a humoral immune response, without the involvement of T helper cells (Th cells) or antigen-presenting cells (APC). Thymus-dependent antigens require $CD4^+$ T cells to induce humoral immune responses. Activated B cells differentiate to plasma cells that secrete large amounts of antibodies, which travel through the bloodstream and tissues to inactivate pathogens and toxins.

The T lymphocyte population consists of different types of T cells. $CD8^+$ cytotoxic T cells (CTL) are a sub-group of T cells that induce the death of cells infected with viruses or other pathogens, or other dysfunctional host cells. Naive $CD8^+$ T cells are activated when their T-cell receptor (TCR) strongly interacts with a peptide-MHC class I complex presented by the host APC. Activated effector CTL can migrate to the site of infection or cancer where, upon recognition of the presented MHC-peptide, it releases perforin and granzyme to induce cell death and lysis of the target cells.

$CD4^+$ T (helper) cells can develop in several types of immune-stimulating and immune-suppressive T cells. Naïve T helper lymphocytes can differentiate into several different directions, thereby governing the right type of immune response. The direction of differentiation depends on the cytokines they are exposed to during their activation [36, 37]. Type 1 T helper (Th1) cells which produce IFNγ and TNF-α, and type 17 T helper cells (Th17) which

produce IL-17A, IL-17F, IL-17AF, IFNγ, IL-21 and IL-22 are important for the control of viral infections and cancer [38, 39]. Type 2 T helper (Th2) cells are the host immunity effectors against extracellular pathogens. Type 2 T helper cells are more commonly associated with asthma and allergic responses, and are characterized by high levels of IL-4, IL-5, IL-9, IL-13 and/or IL-25 [40]. $CD4^+$ T cells can also develop into T regulatory cells (Tregs), either directly in the thymus, where they start to express the transcription factor FOXP3, or in the periphery where they can produce cytokines such as IL-10 and TGFβ [38]. T regulatory cells are important for maintaining peripheral tolerance, which play important role in preventing autoimmune diseases and limiting chronic inflammatory diseases, but can also can suppress sterilizing immunity and limit tumor immunity [41].

Type 1 T helper 1 cells are the host immune effectors against intracellular pathogens such as bacteria and viruses. They secrete interferon-γ (IFNγ) and tumor necrosis factor-α (TNF-α), and activate macrophages to kill microbes. They may also stimulate B cells to secrete specific subclasses of IgG antibodies which can coat extracellular microbes and activate complements. Type 1 T helper effector cells use two signals to activate dendritic cells (DCs). They secrete IFNγ, which binds to IFNγ receptors on the DC surface, and display the costimulatory protein CD40 ligand, which binds to CD40 on DCs. As a result, the DCs increase the expression of MHC classes I and II, B7-family costimulatory proteins and the production of various pro-inflammatory cytokines, especially IL-12, which makes them more effective in stimulating $CD8^+$ T cells to differentiate into effector cells. In addition, they can help to recruit $CD8^+$ T cells and sustain their local proliferation and effector function by the production of IL-2 [42, 43]. Secretion of IFNγ furthermore increases the expression of the TAP transporter proteins and MHC class I molecules in target cells, thereby rendering intracellular pathogen-infected or cancer cells more visible to the $CD8^+$ T cells [44]. Effector Th1 cells can sometimes directly kill target cells by expressing Fas ligand on its surface [38, 45].

2.3 IFNγ signal transduction pathway and downstream effects

IFNγ is the only type II interferon and is produced by T lymphocytes and natural killer (NK) cells [46]. The IFNγ receptor is expressed on many cell types. Both heterodimers of IFNγR1 and IFNγR2 are required for IFNγ signaling. The IFNγR1 subunit of the receptor complex is associated with Jak1, while the IFNγR2 subunit is associated with Jak2. Activation of Jak1 and Jak2 results in phosphorylation of the receptor, following that Jak1 and Jak2 recruit and lead to the phosphorylation of STAT1. Phosphorylated STAT1 form the homodimerization and translocate into nuclear. .In the nucleus, STAT1 homodimers bind to IFNγ-activated sequence (GAS) elements in the promoters of target genes which regulate the transcription, including many transcription factors that activate expression of secondary response genes. . In addition, IFNγ signaling can also activate NF-kappa B, MAPK, PI3K-Akt signaling pathways to regulate the expression of manyother genes (Figure 1).

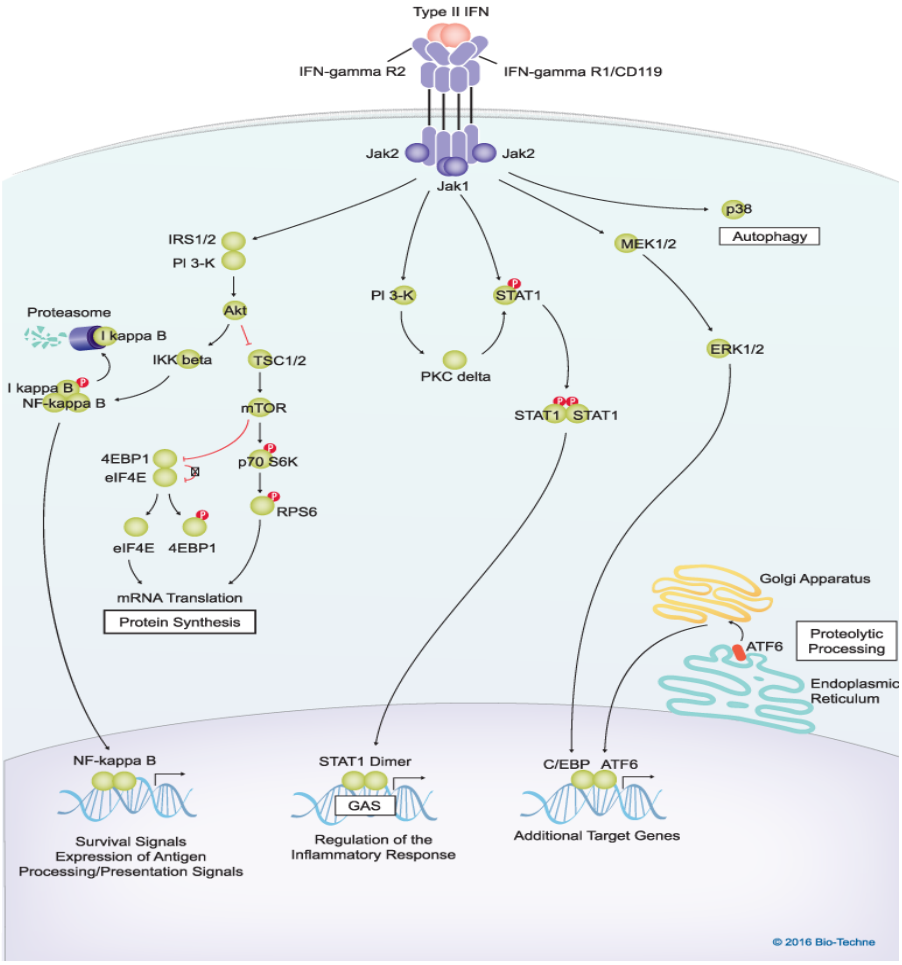
IFNγ is known for its cytotoxic, cytostatic and anti-tumor properties, and mediates its antiproliferative effects by influencing the genes/proteins involved in regulating cell proliferation. IFNγ upregulates p21 and p27 (the cyclin-dependent kinase [CDK] inhibitors) which inhibit the activity of CDK2 and CDK4, respectively, causing cell-cycle arrest at the G1/S checkpoint [47-51]. This IFNγ-mediated inhibition results in suppression of retinoblastoma (Rb) phosphorylation, thereby increasing the levels of the active (E2F-repressing) form of Rb and preventing transcription of E2F-dependent genes required for the S phase [52]. P202, which is upregulated by IFNγ, is a strong cell-cycle repressor that can bind to E2F and inactivate its DNA-binding activity, thereby preventing transcription of E2F-dependent genes required for the S phase [53, 54]. *RARRES1* is the tumor-suppressor gene, which has antiproliferative effects on the cells [55, 56]. The expression of RARRES1 and STAT1 is IFN-dependent [57]. IFNγ induces the expression of interferon-induced transmembrane protein 1 (IFITM1), which plays a key role in IFNγ's anti-proliferative action [58].

IFNγ has also apoptotic effects in an indirect way. It may promote cellular sensitivity to the pro-apoptotic effects of TNFα by promoting the surface expression of a TNFα receptor on tumor cells [59]. IFNγ increases cellular sensitivity to apoptosis by upregulating the expression of Fas and Fas ligand[60, 61]. PKR is an antiviral enzyme that is upregulated by IFNγ and implicated in NFκB activation, TNFα mRNA regulation, TNFα-induced apoptosis and regulation of STAT1 and STAT3 activity [62-66]. The mechanisms by which PKR affects apoptosis are still unclear but may involve induction of Fas [67]. In addition, IFNγ promotes tumor-suppressive effects by increasing the expression of IRF-1, which is a tumor-suppressor gene. IRF-1 is required for the induction of apoptosis by signals like DNA damage. The proapoptotic effects of IRF-1 are mainly mediated by the IRF-1-induced caspase 1 [68-70].

IFNγ also has immunomodulatory effects. IFNγ promotes macrophage activation, drives the development and activation of Th1 cells, enhances NK-cell activity and induces cells to produce chemokines that promote effector-cell trafficking to sites of inflammation [46]. For instance, CXCL9 and CXCL10 are chemoattractants for T cells, the production of which can be increased by IFNγ [53, 71, 72]. Also CCL5 is known to attract memory CD4+ T cells, monocyte and macrophages, which are upregulated by IFNγ [53, 73]. In addition, IFNγ upregulates the production of IL-12 by APC, an important cytokine that drives the differentiation and activation of NK cells and type 1 $CD4^+$ and $CD8^+$ T cells [74, 75].

Several viruses encode proteins that are designed to interfere with type I and II IFN signaling and thereby counteract anti-viral immunity [76, 77]. For instance, hepatitis B virus produce trans-acting factor which inhibit IFNβ synthesis [78], and the HPV16 viral oncoproteins interfere with IFNβ signaling. The E6 protein binds to IRF-3 and blocks its ability to activate the IFNβ gene [79], while the E7 protein binds to IRF-1 through the transactivation domain of IRF-1 [80], thereby

inhibiting the activation of the IFNβ promoter. The vaccinia viral B18R protein can bind the IFNβ of many species [81]. Expression of HCV proteins blocks IFNα-induced gene expression, as well as the formation of ISGF3 and different STAT dimers [82]. The viral protein RIF of Kaposi's sarcoma-associated herpesvirus (KSHV) efficiently blocks type I IFN signaling by inhibition of Tyk2 and JAK1, resulting in impaired response of IFN [83]. Furthermore, respiratory syncytial virus (RSV) inhibits IFN-mediated signaling mechanisms in epithelial cells by impairing IFN-beta-mediated STAT-1 phosphorylation and reducing the nuclear STAT1 interaction with the transcriptional coactivator CBP [84]. Ebola virus VP24 binds karyopherin alpha1 and blocks STAT1 nuclear accumulation, thereby blocking the cellular production of alpha/beta interferon (IFN-alpha/beta) and cells' ability to respond to IFN-alpha/beta or IFN-gamma [85]. The HIV-1 transactivator protein Tat is one of the retroviral proteins identified as a key immune-modulator in AIDS pathogenesis. Tat impairs the STAT1 activation via Tat-dependent induction of the suppressor of cytokine signaling-2 (SOCS-2) activity [86]. Human CMV (HCMV) also inhibits IFN-gamma induced STAT1 tyrosine phosphorylation mediated by SHP2, resulting in impaired IFN-gamma-induced immune response [87]. Finally, the myxoma virus MT-7 protein can serve as a decoy receptor to bind IFNγ [88].



https://www.rndsystems.com/cn/pathways/type-ii-interferon-signaling-pathways

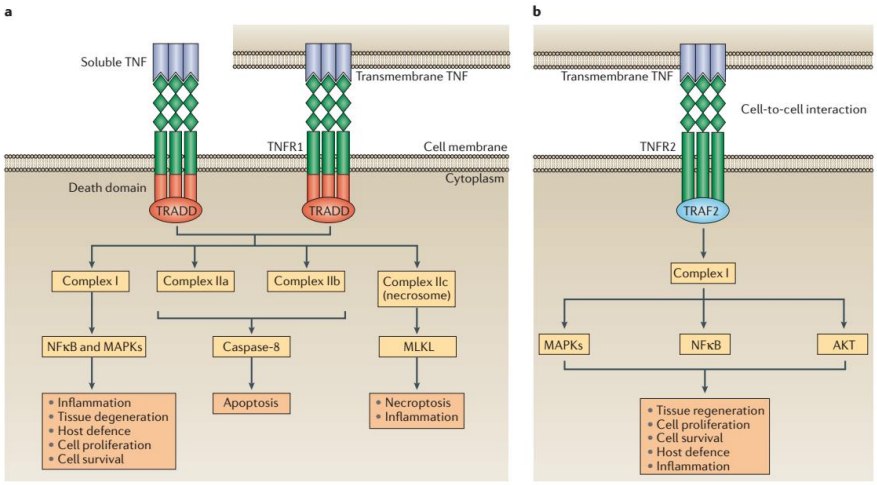
Figure 1 Type II interferon signaling pathway. It has immunomodulatory and antiviral functions. The biologically active form of IFNγ is a non-covalently-linked homodimer, which binds to the extracellular domain of two IFN-gamma R1/CD119 chains. The IFN-gamma receptor complex is composed of IFN-gamma R1 interacts and R2. The IFN-gamma R1 subunits are associated with Jak1 and the IFN-gamma R2 subunits of the receptor complex are associated with Jak2. Activation of Jak1 and Jak2 leads to the phosphorylation of the

receptor and then recruit and phosphorylated STAT1. Phosphorylated STAT1 formshomo-dimersn and translocate into nuclear. In nuclear STAT1 homodimers bind to IFNγ-activated sequence (GAS) elements and regulate the transcription of target genes. In addition, IFNγ signaling can activate MAPK, PI3K-Akt, and NFκB signaling pathways to regulate the expression of many other genes.

2.4 TNFα signal transduction pathway and downstream effects

TNFα is a transmembrane 26 KDa protein expressed by activated monocytes and macrophages and activated NK and T cells, non-immune cells such as endothelial cells and fibroblasts can also express TNFα [89, 90]. TNFα is transcriptionally regulated and induced by transcription-factors such as nuclear factor-kB (NFκB), activator protein-1 (AP1), c-Jun, and nuclear factor associated with activated T cells (NFAT), which bind the promoter region of the TNF gene [91].

soluble TNF or transmembrane TNF bind to the ligation of TNFR1 which leads to the assembly of complex I, Complex I activates nuclear factor κB (NFκB) and mitogen-activated protein kinases (MAPKs) which play crucial role in inflammation and tissue degeneration, maintaining cell survival and proliferation and initiating the immune defense against pathogens (Figure 2a). The formation of the complexes IIa and IIb results in apoptosis, while complex IIc results in necroptosis and inflammation (Figure 2a). TNF receptor 2 can be activated primarily by transmembrane TNF through cell-to-cell interactions. It recruits TNFR-associated factor 2 (TRAF2) via TRAF domain which trigger the formation of complex I and the downstream activation of NFκB, AKT and MAPKs . TNF receptor 2 mainly mediates homeostatic bioactivities, including cell survival, cell proliferation and tissue regeneration .It can also initiate inflammatory effects and the host's defense against pathogens (Figure 2b) [92].



[92]

Figure 2 a | Both soluble and transmembrane TNF can activate TNF receptor 1 (TNFR1) signaling. A death domain of TNFR1 recruits the adaptor protein TNFR1-associated death domain protein (TRADD). Soluble TNF or transmembrane TNF bind to the ligation of TNFR1 leads to the assembly of complex I. Complex I activates NFκB and MAPKs which induces inflammation, cell survival and proliferation, tissue degeneration, and orchestrates the immune defence against pathogens. Alternatively, the formation of the complexes IIa and IIb (also known as ripoptosome) results in apoptosis.Complex IIc (necrosome) induces necroptosis and inflammation. b | TNFR2 can be fully activated primarily by transmembrane TNF through cell-to-cell interactions. TNFR-associated factor 2 (TRAF2) is recruited to TNFR2 via its TRAF domain, resulting in the formation of complex I and activation of AKT , MAPKs and NFκB. TNFR2 mediates mainly homeostatic bioactivities including cell proliferation, cell survival and tissue regeneration. TNFR2 pathway can also initiate inflammatory effects and host defense against pathogens.

TNF-induced cell death signaling is executed by TNFR1 [93], which requires death signaling inducing signaling complex (DISC) proteins,

which include the Fas-associated protein with death domain (FADD), TNFR-associated death domain protein (TRADD), and the TNFR-associated factor-1 (TRAF1) [94-97]. These proteins recruit pro-caspase-8, cleavage of caspase-8 results in its activation and initiates apoptosis [98]. Caspase-8 subsequently processes pro-caspase-3, -6, -7 and other cytosolic substrates, converting these pro-caspases into active enzymes [99]. In particular, activation of caspase-3 is important for TNF-induced cell death. Caspase-3 targets DNase to degrade genomic DNA [100], thus executing cell apoptosis. The protease activity of caspase-8 is tightly regulated by the negative inhibitor protein FLICE/caspase-8 inhibitory protein (cFLIP). The inhibitors of apoptosis proteins (IAPs) are essential regulators of TNFR-induced cell death via their interaction with TRAF2 [101, 102]. Thus, TNF interaction with TNFR1 induces a caspase-dependent apoptosis which is critically regulated by IAPs and cFLIP [103]. Apoptosis is a highly regulated and controlled process leading to programmed cell death. The morphology of apoptosis includes cell shrinkage, chromatin condensation, DNA/nuclear fragmentation and membrane blebbing. Unlike necrosis, apoptosis does not release the intracellular content into the micromilieu, thereby preventing the induction of massive inflammation [104].

Regulated necrosis, termed necroptosis, is a newly discovered pathway of necrosis and can be triggered by members of the tumor necrosis factor (TNF) family [105], TLRs (TLR3 and TLR4) [106-108] and DNA and/or RNA sensors (DAI and possibly RIG-I and MDA5). TNF-facilitated necroptosis requires the inhibition of caspase-8 and the assembly of RIPK1-RIPK3 complex IIb, also called the necrosome [109]. Necroptosis can trigger inflammation as it leads to rapid plasma membrane permeabilization and release of cell contents. This exposes the immune system to damage-associated molecular patterns (DAMPs), including cytokines and alarmins that are released mainly by dying cells (e.g., the interleukin-1 family cytokines IL-1α and IL-33), as well as the S100 proteins S100A8, S100A9 and S100A12. In addition, several cellular components such as ribonucleoproteins,

histones and heat shock proteins released by dying and damaged cells act as DAMPs [110].

Viruses have evolved mechanisms to suppress cell death. For instance, molluscum contagiosum virus (MCV), Kaposi's sarcoma-associated herpesvirus (KSHV) and equine herpesvirus 1 (EHV-1) developed the ability to inhibit caspase-8, thus inhibiting necroptosis. Murine cytomegalovirus counteracts necroptosis by blocking RHIM-dependent signaling pathways and herpes simplex virus (HSV) prevents necroptosis by inhibiting the interaction between RIP1 and RIP3 [111].

2.5 Human papillomavirus-specific T-cell immunity

The T-cell-mediated immune response is important in controlling hrHPV infections. In immunosuppressed individuals, such as transplanted or HIV-infected patients, compromised $CD4^+$ T-cell function is associated with progression of infections to malignancies [112-115]. In immune-competent individuals, asymptomatic HPV-16 infections are associated with the detection of HPV-16-specific Th1 $CD4^+$ T-cell responses [116, 117]. Similarly, spontaneous regression of HPV-induced condyloma is associated with a dense infiltration of both CD4+ and CD8+ T lymphocytes [118] with a Th1 cytokine profile [119, 120]. Furthermore, a predominance of Th1 cytokines is observed in regressing lesions [121, 122]. Proliferative $CD4^+$ type 1 T-cell responses are also associated with spontaneous regression of CIN3 [123]. The role of the HPV-specific T-cell response in clearing HPV infections is supported by data from therapeutic vaccine trials. VGX-3100, which drives the induction of robust HPV16- and HPV18-specific cellular and humoral immune responses, is the first therapeutic vaccine to show efficacy against HPV-induced CIN2/3 [124, 125]. In addition, regression of HPV16-induced grade 3 vulvar-intraepithelial neoplasia was achieved by vaccination with a synthetic long-peptide vaccine against the HPV16 oncoproteins E6 and E7.

Patients with a complete response at three months had a significantly stronger HPV-specific IFNγ-associated T-cell response than patients without a complete clinical response [126-128].

Also in cancer, T cells play an important role. The progression towards invasive cancers features a decrease of CD4+ T-cell infiltrate, an increase of CD8+ T lymphocytes [129-131], a loss of anti-HPV-16 $CD4^+$ T-cell activity [132, 133] and a shift to Th2 type cytokines [134, 135]. Patients with HPV16-associated oropharyngeal squamous cell carcinoma (OPSCC) have a better prognosis than those with a carcinoma not caused by HPV [136, 137]. The results are partly due to the enhanced T-cell infiltration of HPV-positive tumors [138-140]. Similar evidence is found for T cells in cervical cancer [141, 142]. In OPSCC, increased peripheral blood levels of CD8 T lymphocytes predicted an improved overall survival [143], while in cervical cancer the presence of circulating HPV-specific T cells were associated with a better clinical outcome [144]. Unfortunately, these responses are counteracted by several factors. Tregs strongly inhibit the activation, proliferation and cytokine production of $CD4^+$ and $CD8^+$ T cells [145], and have been found in premalignant lesions, early stages of tumor progression and in the draining lymph nodes [146-148]. The numbers of TGF-β-producing Treg display a positive correlation with lesion progression [149] and the number of $Foxp3^+$ Treg cells in CIN lesions, VIN lesions and cancer are associated with shorter recurrence-free or overall survival [150, 151]. Furthermore, IL-10 produced by tumor macrophages induces regulatory phenotype on T cells [152]. Some of the Tregs are activated upon recognition of HPV antigens. HPV16-specific regulatory T cells have been found in CIN3 lesions, cervical cancers and OPSCC [128, 133, 147]. In addition, HPV-specific immunity can be counteracted by immune-suppressive myeloid cells. Upon tumor progression, the number of systemic and local immune-suppressive myeloid cells increases in patients or mice with HPV-induced cancers [153-155].

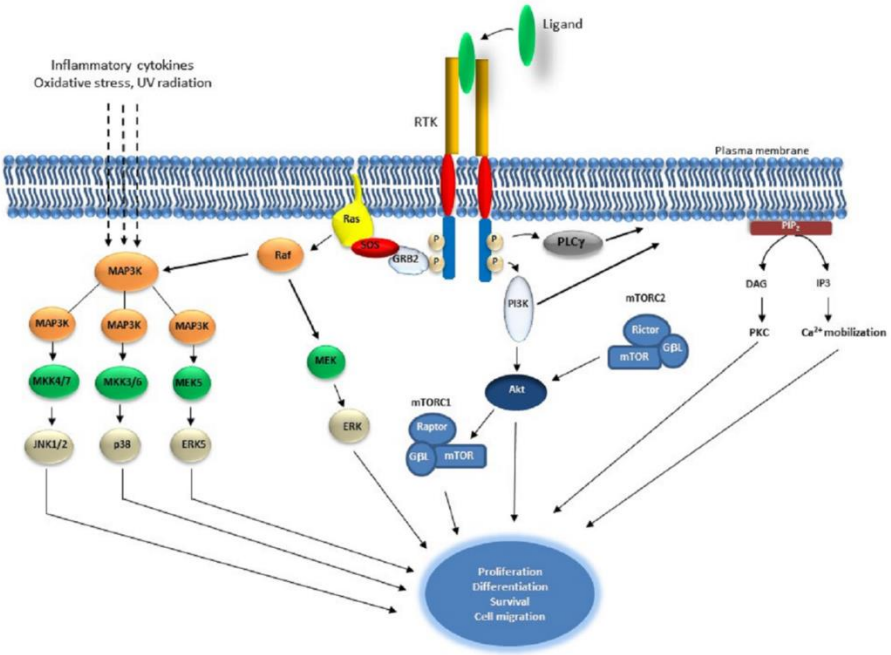
HPV-positive cervical cancer cells and HPV-E6 overexpressing cells can inhibit monocyte differentiation to DC in an IL-6-dependent manner

[156-158], as a result of which inflammatory monocytes/macrophages infiltrating the tumor become tolerogenic or M2 type of macrophages [156, 159] and accumulate in the tumor [156]. As the functional phenotype of myeloid cells can change upon receiving different environmental cues, including the presence of IFNγ-producing T cells [159]. It is very important to induce a strong HPV-specific cellular immune response, including $CD4^+$, $CD8^+$ T cells, as well as Th1 type cytokines in the therapeutic approach of HPV-related cancer. Infiltration with appropriately stimulated myeloid cells fosters a better clinical outcome [160] and the regression of tumors [153, 161].

3. Epidermal growth factor receptor signaling

The epidermal growth factor receptor (EGFR) family plays an important role in cell growth. The EGFR family of receptor tyrosine kinases comprises the EGFRs ErbB1, ErbB2/HER2/neu, ErbB3/HER3 and ErbB4/HER4. Many cancers have a mutated or overexpressed EGFR. Activating mutations and overexpression of EGFR family members contribute to oncogenesis by inducing proliferation and resistance to apoptosis.

EGF binds to its receptors thereby triggering the phosphorylation of the receptor subunits which allow proteins to bind through Src homology 2 (SH2) domains, leading to the activation Of downstream signaling including the PI3K-AKT-mTOR pathways and RAS-RAF-ERK (Figure 3).



[162]

Figure 3 EGFR and downstream signaling pathways. Ligand binds to the receptor which trigger tyrosine kinases auto-phosphorylation, resulting in the activation of Ras and induction of serine/threonine kinase Raf. Following the activation of Raf, Mek1/2 are phosphorylated which in turn phosphorylate and activate Erk1/2. Raf also activates MAP3 kinases which activate MKK4/7, MKKK3/6 and MEK5, resulting in activation of JNK1/2, p38 and ERK5, consecutively. Inflammatory cytokines, oxidative stress and UV radiation can also activate MAP3Ks pathways.. Auto-phosphorylation EGFR activates PI3K and results in the activation of Akt. Aktinduces mTOR within the mTORC1 complex and Akt is also regulated by mTORC2 complex. PLCγ activation leads to Ca^{+2} mobilization as well as the activation of PKC. EGFR signaling plays an important role in differentiation, survival, proliferation, and cell migration.

After the binding of Grb2 or Shc to phosphorylated ErbB receptors, epidermal growth factor activates the ERK pathway which results in the recruitment of the son of sevenless (SOS) to the activated receptor dimer [163]. SOS starts a cascade by activating RAS, which activates RAF-1 to phosphorylate MEK1/2, resulting in the activation of ERK1/2. This pathway is known to stimulate cell proliferation and promote cell survival (Figure 3).

Epidermal growth factor also motivates cell survival through the activation of PI3k/AKT signaling (Figure 3) [163]. Recruitment of PI3 kinase to activated ErbB receptors is mediated by the binding of SH2 domains in PI3 kinase to phosphorylated tyrosine residues. Following that, the catalytic subunit of PI 3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P2) and leads to the formation of PtdIns(3,4,5)P3. PI3-kinase can also activate RAS-ERK signaling, which plays important role in cell survival. AKT (PKB) whichis a key downstream effector of PtdIns(3,4,5)P3 promotes cell survival by activating the transcription of anti-apoptotic proteins. NFκB and CREB are also involved in this process. Glycogen synthase kinase 3 (GSK3) is another downstream target of AKT, the constitutive activation of GSK3 leads to the phosphorylation and inhibition of guanine nucleotide exchange factor eIF2B that initiates protein translation. As a consequence, the inactivation of GSK3 by AKT result in the promotion of protein synthesis [164]. AKT also activates the mammalian target of rapamycin (mTOR), which plays a role in promoting protein synthesis through p70 ribosomal S6 kinase (p70s6k) and inhibition of the eIF-4E binding protein (4E-BP1) [165]. Collectively, these processes promote cell growth and survival in response to EGF.

Dysregulation of EGFR has been observed in a wide variety of carcinomas, including head and neck, breast, bladder, ovarian, renal, colon and NSCLC [166]. Mutations or amplification of EGFR are associated with a number of cancers, including squamous cell carcinoma of the lung (80% of cases), anal cancers, glioblastoma (50%) and epithelial tumors of the head and neck (80–100%) [167, 168]. The

overexpression of EGFR is mostly due to EGFR-gene amplification or mutations. EGFR mutationsoccurrs frequently in EGFR exons 18–21, which encode the tyrosine kinase section of the EGFR protein [169-171]. The most common truncation mutant of EGFR is EGFRvIII, in which amino acids 6–273 (encoded by exons 2–7) are deleted from the gene [172]. These somatic mutations lead to constant EGFR activation and result in uncontrolled cell division.

In addition, several studies show an impact of EGFR overexpression on the immune response [173]. An association between mutant EGFR and increased PD-L1 expression has been reported. Epidermal growth factor receptor activation by EGF stimulation or mutant EGFR-upregulated PD-L1 expression occurs by activating the PI3K-AKT and MEK-ERK signaling pathways in NSCLC cells [174-176]. Mutant EGFR signaling drives increased PD-L1 expression on EGFR-driven murine lung tumors. Blockade of PD1 has been found to improve the survival of tumor-bearing mice [177]. In addition, overexpression of mutant EGFR induced PD-L1 expression on human bronchial epithelial cell lines [177] and the use of EGFR inhibitors reduced PD-L1 expression in NSCLC cell lines [177]. In addition, EGFR has been found to inhibit the phosphorylation of STAT1 mediated by SHP2, which may impair the response of malignant cells to IFNs [178]. EGFR signaling is involved in several cellular processes promoting malignancy. Several means to target and interfere with the effects of EGFR signaling have been developed. Currently, there are two distinct therapeutic approaches to target EGFR: a) monoclonal antibodies, including cetuximab and panitumumab, and b) small molecule tyrosine kinase inhibitors, such as gefitimnib, erlotinib, lapatinib and canertinib [179]. Both monoclonal antibodies (mAbs), cetuximab and panitumumab, activate NK cells. Cetuximab-activated neutrophils mediate antibody-dependent cellular cytotoxicity (ADCC) against head and neck squamous cell carcinomas (HNSCC) tumor cells. Cetuximab also enhances DC maturation [180]. Thus, EGFR signaling also has effects on immune response.

4 Scope of this thesis

In this thesis, we study the mechanisms that allow HPV-infected or -transformed cells to resist the HPV-specific Th1 type of immune response. Chapter 2 discusses the use of a system resembling the natural infection with HPV as closely as possible. It comprised primary KCs that stably maintained the hrHPV genome as episomes and that were shown to undergo the entire differentiation-dependent HPV life cycle in organotypic raft cultures. In addition, non-infected primary KC cultures were used as well as primary KCs that were newly infected with authentic HPV16 virions. Human papillomavirus downregulated the key protein RIKP3 for necroptosis to escape the necroptosis induced by the the cytokines IFNγ and TNFα of type 1 T cells. Global histone methyltransferase inhibitor 3-deazaneplanocin (DZNeP) restored the expression of RIPK3 and increased necroptosis in hrHPV-positive KCs. Simultaneously, HPV effectively inhibited the IFNγ and TNFα-mediated arrest of cell growth at the S-phase by downregulating *IFITM1*, followed by an increase in the expression of the antiproliferative gene *RARRES1* and a decrease in the proliferative gene *PCNA*. In chapter 3, we review how HPV interferes with type I and II IFNs, as well as the TNFα and CD40 signaling routes, and describe the impact of this on the induction of HPV-specific immunity and the resistance to vaccine-induced HPV-specific T cells. In chapter 4, we focus on HPV-positive head and neck cancer. We show that, similar to HPV-infected KCs, also HPV-induced tumor cells lacked sensitivity to IFNγ and TNFα-induced necroptosis by a lack of RIPK3 expression. However, patients with type 1 HPV16-specific T cells have a better overall survival, a smaller tumor size and less lymph-node metastases. Moreover, we found that IFNγ and TNFα, and the supernatant of Th1 and Th17 cultures induced growth arrest in the OPSCC cell lines and that this was associated with the increased expression of both the anti-proliferative genes, *IFITM1* and *RARRES1*. This suggests that a strong type 1 T-cell response may partly overcome the escape mechanisms. Furthermore, we argue that it would be beneficial to keep cisplatin chemotherapy within the treatment, as the type 1 cytokines IFNγ and TNFα synergized with

platinum-based chemotherapy in killing tumor cells. Resistance to antitumor immunity can be promoted by the oncogenic pathways operational in human cancers, including the EGFR pathway. In chapter 5, we examine whether and how EGFR downstream signaling in HNSCC can affect the attraction of immune cells. Stimulation of HNSCC cells with IFNγ and TNFα triggered the production of T-cell-attracting chemokines and required c-RAF activation. Blocking the EGFR with cetuximab increased chemokine production and augmented the attraction of T cells. Mechanistic studies reveal that overexpression of the EGFR results in the activation of the MEK- and JNK-signaling pathways that act simultaneously to suppress the production of chemokines required to attract T cells. Finally, chapter 6 presents a general discussion about the effects of type 1 T cells on HPV-infected cells and HPV-induced cancers, followed by the conclusion of this thesis.

Reference

1. Haedicke J, Iftner T. Human papillomaviruses and cancer. Radiotherapy and Oncology. 2013;108(3):397-402.

2. PINIDIS P, TSIKOURAS P, IATRAKIS G, ZERVOUDIS S, KOUKOULI Z, BOTHOU A, et al. Human Papilloma Virus' Life Cycle and Carcinogenesis. Maedica-a Journal of Clinical Medicine. 2016;11(1).

3. Johansson C, Schwartz S. Regulation of human papillomavirus gene expression by splicing and polyadenylation. Nature reviews Microbiology. 2013;11(4):239.

4. Ajila V, Shetty H, Babu S, Shetty V, Hegde S. Human papilloma virus associated squamous cell carcinoma of the head and neck. Journal of sexually transmitted diseases. 2015;2015.

5. De Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. The lancet oncology. 2012;13(6):607-15.

6. Jemal A, Simard EP, Dorell C, Noone A-M, Markowitz LE, Kohler B, et al. Annual report to the nation on the status of cancer, 1975–2009, featuring the burden and trends in human papillomavirus (HPV)–associated cancers and HPV vaccination coverage levels. JNCI: Journal of the National Cancer Institute. 2013;105(3):175-201.

7. Control CfD, Prevention. Prevention of genital HPV infection and sequelae: report of an external consultants' meeting. Atlanta: Centers for Disease Control and Prevention. 1999.

8. Winer RL, Hughes JP, Feng Q, O'reilly S, Kiviat NB, Holmes KK, et al. Condom use and the risk of genital human papillomavirus infection in young women. New England Journal of Medicine. 2006;354(25):2645-54.

9. Zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nature reviews cancer. 2002;2(5):342.

10. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. Journal of clinical oncology. 2011;29(32):4294-301.

11. Henneman R, Van Monsjou HS, Verhagen CV, Van Velthuysen M-LF, Ter Haar NT, Osse EM, et al. Incidence changes of human papillomavirus in oropharyngeal squamous cell carcinoma and effects on survival in the Netherlands Cancer Institute, 1980-2009. Anticancer research. 2015;35(7):4015-22.

12. Gillison ML, Chaturvedi AK, Lowy DR. HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. Cancer. 2008;113(S10):3036-46.

13. Broz P, Monack DM. Newly described pattern recognition receptors team up against intracellular pathogens. Nature Reviews Immunology. 2013;13(8):551-65.

14. Baker B, Ovigne JM, Powles A, Corcoran S, Fry L. Normal keratinocytes express toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. British Journal of Dermatology. 2003;148(4):670-9.

15. Lebre MC, van der Aar AM, van Baarsen L, van Capel TM, Schuitemaker JH, Kapsenberg ML, et al. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. Journal of Investigative Dermatology. 2007;127(2):331-41.

16. Miller LS. Toll-like receptors in skin. Advances in dermatology. 2008;24:71.

17. Mempel M, Voelcker V, Köllisch G, Plank C, Rad R, Gerhard M, et al. Toll-like receptor expression in human keratinocytes: nuclear factor κB controlled gene activation by Staphylococcus aureus is Toll-like receptor 2 but not Toll-like receptor 4 or platelet activating factor receptor dependent. Journal of Investigative Dermatology. 2003;121(6):1389-96.

18. Begon É, Michel L, Flageul B, Beaudoin I, Jean-Louis F, Bachelez H, et al. Expression, subcellular localization and cytokinic modulation of Toll-like receptors (TLRs) in normal human keratinocytes: TLR2 up-regulation in psoriatic skin. European Journal of Dermatology. 2007;17(6):497-506.

19. Lebre MC, Antons JC, Kalinski P, Schuitemaker JH, Van Capel TM, Kapsenberg ML, et al. Double-stranded RNA-exposed human keratinocytes promote Th1 responses by inducing a type-1 polarized phenotype in dendritic cells: role of keratinocyte-derived tumor necrosis factor α, type I interferons, and interleukin-18. Journal of Investigative Dermatology. 2003;120(6):990-7.

20. Prens EP, Kant M, Van Dijk G, Van Der Wel LI, Mourits S, Van Der Fits L. IFN-α enhances poly-IC responses in human keratinocytes by inducing expression of

cytosolic innate RNA receptors: relevance for psoriasis. Journal of Investigative Dermatology. 2008;128(4):932-8.

21. Köllisch G, Kalali BN, Voelcker V, Wallich R, Behrendt H, Ring J, et al. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. Immunology. 2005;114(4):531-41.

22. Miller LS, Modlin RL. Human keratinocyte Toll-like receptors promote distinct immune responses. Journal of Investigative Dermatology. 2007;127(2).

23. Sunthamala N, Thierry F, Teissier S, Pientong C, Kongyingyoes B, Tangsiriwatthana T, et al. E2 proteins of high risk human papillomaviruses down-modulate STING and IFN-kappa transcription in keratinocytes. PloS one. 2014;9(3):e91473. doi: 10.1371/journal.pone.0091473. PubMed PMID: 24614210; PubMed Central PMCID: PMC3948877.

24. Karim R, Tummers B, Meyers C, Biryukov JL, Alam S, Backendorf C, et al. Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response. PLoS Pathog. 2013;9(5):e1003384. doi: 10.1371/journal.ppat.1003384. PubMed PMID: 23717208; PubMed Central PMCID: PMC3662672.

25. Pacini L, Savini C, Ghittoni R, Saidj D, Lamartine J, Hasan UA, et al. Downregulation of Toll-Like Receptor 9 Expression by Beta Human Papillomavirus 38 and Implications for Cell Cycle Control. Journal of virology. 2015;89(22):11396-405. doi: 10.1128/JVI.02151-15. PubMed PMID: 26339055; PubMed Central PMCID: PMC4645680.

26. Yu SL, Chan PK, Wong CK, Szeto CC, Ho SC, So K, et al. Antagonist-mediated down-regulation of Toll-like receptors increases the prevalence of human papillomavirus infection in systemic lupus erythematosus. Arthritis research & therapy. 2012;14(2):R80. doi: 10.1186/ar3803. PubMed PMID: 22513098; PubMed Central PMCID: PMC3446454.

27. Hasan UA, Bates E, Takeshita F, Biliato A, Accardi R, Bouvard V, et al. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. The Journal of Immunology. 2007;178(5):3186-97.

28. Karim R, Meyers C, Backendorf C, Ludigs K, Offringa R, van Ommen G-JB, et al. Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes. PLoS One. 2011;6(3):e17848.

29. Chiang C, Pauli E-K, Biryukov J, Feister KF, Meng M, White EA, et al. The human papillomavirus E6 oncoprotein targets USP15 and TRIM25 to suppress RIG-I-mediated innate immune signaling. Journal of virology. 2018;92(6):e01737-17.

30. Sunthamala N, Thierry F, Teissier S, Pientong C, Kongyingyoes B, Tangsiriwatthana T, et al. E2 proteins of high risk human papillomaviruses down-modulate STING and IFN-κ transcription in keratinocytes. PloS one. 2014;9(3):e91473.

31. Niebler M, Qian X, Höfler D, Kogosov V, Kaewprag J, Kaufmann AM, et al. Post-translational control of IL-1β via the human papillomavirus type 16 E6 oncoprotein: a novel mechanism of innate immune escape mediated by the E3-ubiquitin ligase E6-AP and p53. PLoS pathogens. 2013;9(8):e1003536.

32. Li S, Labrecque S, Gauzzi MC, Cuddihy AR, Wong AH, Pellegrini S, et al. The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha. Oncogene. 1999;18(42):5727-37. doi: 10.1038/sj.onc.1202960. PubMed PMID: 10523853.

33. Chang YE, Laimins LA. Microarray analysis identifies interferon-inducible genes and Stat-1 as major transcriptional targets of human papillomavirus type 31. Journal of virology. 2000;74(9):4174-82.

34. Tummers B, Goedemans R, Pelascini LP, Jordanova ES, van Esch EM, Meyers C, et al. The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NFkappaB activation. Nat Commun. 2015;6:6537. doi: 10.1038/ncomms7537. PubMed PMID: 26055519; PubMed Central PMCID: PMC4382698.

35. Tummers B, Goedemans R, Jha V, Meyers C, Melief CJ, van der Burg SH, et al. CD40-mediated amplification of local immunity by epithelial cells is impaired by HPV. J Invest Dermatol. 2014;134(12):2918-27. doi: 10.1038/jid.2014.262. PubMed PMID: 24945092; PubMed Central PMCID: PMC4227541.

36. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. Annual review of immunology. 2009;28:445-89.

37. Ziegler SF. Division of labour by CD4+ T helper cells. Nature Reviews Immunology. 2016;16(7):403.

38. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008;112(5):1557-69.

39. Liew FY. T(H)1 and T(H)2 cells: a historical perspective. Nature reviews Immunology. 2002;2(1):55-60. doi: 10.1038/nri705. PubMed PMID: 11905838.

40. Paul WE, Zhu J. How are T H 2-type immune responses initiated and amplified? Nature Reviews Immunology. 2010;10(4):225.

41. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nature Reviews Immunology. 2008;8(7):523.

42. Bos R, Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. Cancer research. 2010;70(21):8368-77.

43. Wong SJ, Bos R, Sherman LA. Tumor-specific CD4+ T cells render the tumor environment permissive for infiltration by low-avidity CD8+ T cells. The Journal of Immunology. 2008;180(5):3122-31.

44. Ma W, Lehner PJ, Cresswell P, Pober JS, Johnson DR. Interferon-γ rapidly increases peptide transporter (TAP) subunit expression and peptide transport capacity in endothelial cells. Journal of Biological Chemistry. 1997;272(26):16585-90.

45. Luckheeram RV, Zhou R, Verma AD, Xia B. CD4(+)T cells: differentiation and functions. Clinical & developmental immunology. 2012;2012:925135. doi: 10.1155/2012/925135. PubMed PMID: 22474485; PubMed Central PMCID: PMC3312336.

46. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. Journal of leukocyte biology. 2004;75(2):163-89.

47. Xaus J, Cardó M, Valledor AF, Soler C, Lloberas J, Celada A. Interferon γ induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. Immunity. 1999;11(1):103-13.

48. Harvat BL, Seth P, Jetten A. The role of p27 Kip1 in gamma interferon-mediated growth arrest of mammary epithelial cells and related defects in mammary carcinoma cells. Oncogene. 1997;14(17):2111.

49. Matsuoka M, Nishimoto I, Asano S. Interferon-γ impairs physiologic downregulation of cyclin-dependent kinase inhibitor, p27Kip1, during G1 phase progression in macrophages. Experimental hematology. 1999;27(2):203-9.

50. Kominsky S, Johnson HM, Bryan G, Tanabe T, Hobeika AC, Subramaniam PS, et al. IFNγ inhibition of cell growth in glioblastomas correlates with increased levels of the cyclin dependent kinase inhibitor p21 WAF1/CIP1. Oncogene. 1998;17(23):2973.

51. Mandal M, Bandyopadhyay D, Goepfert TM, Kumar R. Interferon-induces expression of cyclin-dependent kinase-inhibitors p21 WAF1 and p27 Kip1 that prevent activation of cyclin-dependent kinase by CDK-activating kinase (CAK). Oncogene. 1998;16(2):217.

52. Harvat BL, Jetten A. Gamma-interferon induces an irreversible growth arrest in mid-G1 in mammary epithelial cells which correlates with a block in hyperphosphorylation of retinoblastoma. Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research. 1996;7(3):289-300.

53. Gil MP, Bohn E, O'Guin AK, Ramana CV, Levine B, Stark GR, et al. Biologic consequences of Stat1-independent IFN signaling. Proceedings of the National Academy of Sciences. 2001;98(12):6680-5.

54. Choubey D, Gutterman JU. Inhibition of E2F-4/DP-1-stimulated transcription by p202. Oncogene. 1997;15(3):291.

55. Coyle KM, Vaghar-Kashani A, Wong F, Dean C, Giacomantonio C, Marcato P. RARRES1 is a tumor suppressor in triple-negative breast cancer cell lines. AACR; 2014.

56. Huebner H, Ruebner M, Strissel PL, Schneider-Stock R, Kehl S, Rascher W, et al. Epigenetic and genetic alterations of the placental tumor-suppressor gene rarres1 during human placentogenesis. Placenta. 2014;35(9):A35.

57. Li J, Yu B, Song L, Eschrich S, Haura EB. Effects of IFN-γ and Stat1 on gene expression, growth, and survival in non-small cell lung cancer cells. Journal of Interferon & Cytokine Research. 2007;27(3):209-20.

58. Yang G, Xu Y, Chen X, Hu G. IFITM1 plays an essential role in the antiproliferative action of interferon-γ. Oncogene. 2007;26(4):594.

59. Tsujimoto M, Yip Y, Vilcek J. Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor. The Journal of Immunology. 1986;136(7):2441-4.

60. Xu X, Fu X-Y, Plate J, Chong AS. IFN-γ induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. Cancer research. 1998;58(13):2832-7.

61. Zheng H, Luo R, Zhang L, Mai G. Interferon-gamma up-regulates Fas expression and increases Fas-mediated apoptosis in tumor cell lines. Di 1 jun yi da xue xue bao= Academic journal of the first medical college of PLA. 2002;22(12):1090-2.

62. Meurs E, Chong K, Galabru J, Thomas NSB, Kerr IM, Williams BR, et al. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell. 1990;62(2):379-90.

63. Zamanian-Daryoush M, Mogensen TH, DiDonato JA, Williams BR. NF-κB activation by double-stranded-RNA-activated protein kinase (PKR) is mediated through NF-κB-inducing kinase and IκB kinase. Molecular and cellular biology. 2000;20(4):1278-90.

64. Donzé O, Dostie J, Sonenberg N. Regulatable expression of the interferon-induced double-stranded RNA dependent protein kinase PKR induces apoptosis and fas receptor expression. Virology. 1999;256(2):322-9.

65. Balachandran S, Kim CN, Yeh WC, Mak TW, Bhalla K, Barber GN. Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling. The EMBO journal. 1998;17(23):6888-902.

66. Wong AHT, Tam NWN, Yang YL, Cuddihy AR, Li S, Kirchhoff S, et al. Physical association between STAT1 and the interferon-inducible protein kinase PKR and implications for interferon and double-stranded RNA signaling pathways. The EMBO Journal. 1997;16(6):1291-304.

67. Yeung MC, Liu J, Lau AS. An essential role for the interferon-inducible, double-stranded RNA-activated protein kinase PKR in the tumor necrosis factor-induced apoptosis in U937 cells. Proceedings of the National Academy of Sciences. 1996;93(22):12451-5.

68. Taniguchi T, Lamphier MS, Tanaka N. IRF-1: the transcription factor linking the interferon response and oncogenesis. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 1997;1333(1):M9-M17.

69. TAMURA T, ISHIHARA M, LAMPHIER MS, TANAKA N, OISHI I, AIZAWA S, et al. DNA damage-induced apoptosis and Ice gene induction in mitogenically activated T lymphocytes require IRF-1. Leukemia (08876924). 1997;11.

70. Chin YE, Kitagawa M, Kuida K, Flavell RA, Fu X-Y. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Molecular and cellular biology. 1997;17(9):5328-37.

71. Liao F, Rabin RL, Yannelli JR, Koniaris LG, Vanguri P, Farber JM. Human Mig chemokine: biochemical and functional characterization. Journal of Experimental Medicine. 1995;182(5):1301-14.

72. Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo J, Harada A, et al. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. Journal of Experimental Medicine. 1993;177(6):1809-14.

73. Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. Trends in immunology. 2001;22(2):83-7.

74. Yoshida A, Koide Y, Uchijima M, Yoshida T. IFN-γ induces IL-12 mRNA expression by a murine macrophage cell line, J774. Biochemical and biophysical research communications. 1994;198(3):857-61.

75. Kubin M, Chow J, Trinchieri G. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor alpha, and IL-1 beta production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. Blood. 1994;83(7):1847-55.

76. Khan S, Zimmermann A, Basler M, Groettrup M, Hengel H. A cytomegalovirus inhibitor of gamma interferon signaling controls immunoproteasome induction. Journal of virology. 2004;78(4):1831-42.

77. Alcami A, Smith GL. Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. Journal of virology. 1995;69(8):4633-9.

78. Whitten TM, Quets AT, Schloemer RH. Identification of the hepatitis B virus factor that inhibits expression of the beta interferon gene. Journal of virology. 1991;65(9):4699-704.

79. Ronco LV, Karpova AY, Vidal M, Howley PM. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. Genes & development. 1998;12(13):2061-72.

80. Park J-S, Kim E-J, Kwon H-J, Hwang E-S, Namkoong S-E, Um S-J. Inactivation of interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein Implication for the E7-mediated immune evasion mechanism in cervical carcinogenesis. Journal of Biological Chemistry. 2000;275(10):6764-9.

81. Symons JA, Alcami A, Smith GL. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species soecificity. Cell. 1995;81(4):551-60.

82. Heim MH, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. Journal of Virology. 1999;73(10):8469-75.

83. Bisson SA, Page A-L, Ganem D. A Kaposi's sarcoma-associated herpesvirus protein that forms inhibitory complexes with type I interferon receptor subunits, Jak and STAT proteins, and blocks interferon-mediated signal transduction. Journal of virology. 2009;83(10):5056-66.

84. Senft AP, Taylor RH, Lei W, Campbell SA, Tipper JL, Martinez MJ, et al. Respiratory Syncytial Virus Impairs Macrophage IFN-α/β–and IFN-γ–Stimulated Transcription by Distinct Mechanisms. American journal of respiratory cell and molecular biology. 2010;42(4):404-14.

85. Leung LW, Hartman AL, Martinez O, Shaw ML, Carbonnelle C, Volchkov VE, et al. Ebola virus VP24 binds karyopherin α1 and blocks STAT1 nuclear accumulation. Journal of virology. 2006;80(11):5156-67.

86. Cheng SM, Li JC, Lee DC, Liu L, Chen Z, Lau AS. HIV-1 transactivator protein induction of suppressor of cytokine signaling-2 contributes to dysregulation of IFNγ signaling. Blood. 2009;113(21):5192-201.

87. Baron M, Davignon J-L. Inhibition of IFN-γ-induced STAT1 tyrosine phosphorylation by human CMV is mediated by SHP2. The Journal of Immunology. 2008;181(8):5530-6.

88. Upton C, Mossman K, McFadden G. Encoding of a homolog of the IFN-gamma receptor by myxoma virus. Science. 1992;258(5086):1369-72.

89. Falvo JV, Tsytsykova AV, Goldfeld AE. Transcriptional control of the TNF gene. TNF Pathophysiology. 11: Karger Publishers; 2010. p. 27-60.

90. Tsai EY, Yie J, Thanos D, Goldfeld AE. Cell-type-specific regulation of the human tumor necrosis factor alpha gene in B cells and T cells by NFATp and ATF-2/JUN. Molecular and Cellular Biology. 1996;16(10):5232-44.

91. Tuohy VK, Yu M, Yin L, Kawczak JA, Johnson JM, Mathisen PM, et al. The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. Immunological reviews. 1998;164(1):93-100.

92. Kalliolias GD, Ivashkiv LB. TNF biology, pathogenic mechanisms and emerging therapeutic strategies. Nature Reviews Rheumatology. 2016;12(1):49.

93. Tartaglia LA, Rothe M, Hu Y-F, Goeddel DV. Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. Cell. 1993;73(2):213-6.

94. Hsu H, Shu H-B, Pan M-G, Goeddel DV. TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell. 1996;84(2):299-308.

95. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell. 1995;81(4):495-504.

96. Rothe M, Sarma V, Dixit VM, Goeddel DV. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science. 1995;269(5229):1424-7.

97. Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell. 1994;78(4):681-92.

98. Boldin MP, Goncharov TM, Goltseve YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1-and TNF receptor–induced cell death. Cell. 1996;85(6):803-15.

99. Ho Pk, Hawkins CJ. Mammalian initiator apoptotic caspases. The FEBS journal. 2005;272(21):5436-53.

100. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. 1998;391(6662):43.

101. Vince JE, Pantaki D, Feltham R, Mace PD, Cordier SM, Schmukle AC, et al. TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (TNF) to efficiently activate NF-κB and to prevent TNF-induced apoptosis. Journal of Biological Chemistry. 2009;284(51):35906-15.

102. Wang C-Y, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science. 1998;281(5383):1680-3.

103. Sedger LM, McDermott MF. TNF and TNF-receptors: from mediators of cell death and inflammation to therapeutic giants–past, present and future. Cytokine & growth factor reviews. 2014;25(4):453-72.

104. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology. 2007;35(4):495-516.

105. Vercammen D, Vandenabeele P, Beyaert R, Declercq W, Fiers W. Tumour necrosis factor-induced necrosis versus anti-Fas-induced apoptosis in L929 cells. Cytokine. 1997;9(11):801-8.

106. Chan FK-M, Shisler J, Bixby JG, Felices M, Zheng L, Appel M, et al. A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. Journal of Biological Chemistry. 2003;278(51):51613-21.

107. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, et al. Fas triggers an alternative, caspase-8–independent cell death pathway using the kinase RIP as effector molecule. Nature immunology. 2000;1(6):489.

108. Laster S, Wood J, Gooding L. Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. The Journal of Immunology. 1988;141(8):2629-34.

109. Vandenabeele P, Galluzzi L, Berghe TV, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature reviews Molecular cell biology. 2010;11(10):700.

110. Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. Nature. 2015;517(7534):311.

111. Guo H, Omoto S, Harris PA, Finger JN, Bertin J, Gough PJ, et al. Herpes simplex virus suppresses necroptosis in human cells. Cell host & microbe. 2015;17(2):243-51.

112. Arends M, Benton E, McLaren K, Stark L, Hunter J, Bird C. Renal allograft recipients with high susceptibility to cutaneous malignancy have an increased prevalence of human papillomavirus DNA in skin tumours and a greater risk of anogenital malignancy. British journal of cancer. 1997;75(5):722.

113. Chirgwin KD, Feldman J, Augenbraun M, Landesman S, Minkoff H. Incidence of venereal warts in human immunodeficiency virus-infected and uninfected women. Journal of Infectious Diseases. 1995;172(1):235-8.

114. Sun X-W, Kuhn L, Ellerbrock TV, Chiasson MA, Bush TJ, Wright Jr TC. Human papillomavirus infection in women infected with the human immunodeficiency virus. New England Journal of Medicine. 1997;337(19):1343-9.

115. Williams AB, Darragh TM, Vranizan K, Ochia C, Moss AR. Anal and cervical human papillomavirus infection and risk of anal and cervical epithelial abnormalities in human immunodeficiency virus-infected women. International Journal of Gynecology & Obstetrics. 1994;47(1):84.

116. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Research. 2002;62(2):472-9.

117. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer research. 2003;63(3):636-41.

118. Coleman N, Birley HD, Renton AM, Hanna NF, Ryait BK, Byrne M, et al. Immunological events in regressing genital warts. American journal of clinical pathology. 1994;102(6):768-74.

119. Arany I, Tyring SK. Status of Local Cellular Immunity in Interferon-Responsive and-Nonresponsive Human Papillomavirus-Associated Lesions. Sexually transmitted diseases. 1996;23(6):475-80.

120. ARANY I, TYRING SK. Activation of local cell-mediated immunity in interferon-responsive patients with human papillomavirus-associated lesions. Journal of interferon & cytokine research. 1996;16(6):453-60.

121. Scott M, Stites DP, Moscicki A-B. Th1 cytokine patterns in cervical human papillomavirus infection. Clinical and diagnostic laboratory immunology. 1999;6(5):751-5.

122. Xu Y, Zhu KJ, Zhu N, Jiang DH, Chen XZ, Cheng H. Expression of Foxp3+ CD4+ CD25+ regulatory T cells and Th1/Th2, Tc1/Tc2 profiles in the peripheral blood of patients with condyloma acuminatum. Clinical and experimental dermatology. 2009;34(2):229-35.

123. Kadish AS, Timmins P, Wang Y, Ho GY, Burk RD, Ketz J, et al. Regression of cervical intraepithelial neoplasia and loss of human papillomavirus (HPV) infection is associated with cell-mediated immune responses to an HPV type 16 E7 peptide. Cancer Epidemiology and Prevention Biomarkers. 2002;11(5):483-8.

124. Trimble CL, Morrow MP, Kraynyak KA, Shen X, Dallas M, Yan J, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. The Lancet. 2015;386(10008):2078-88.

125. Morrow MP, Kraynyak KA, Sylvester AJ, Shen X, Amante D, Sakata L, et al. Augmentation of cellular and humoral immune responses to HPV16 and HPV18 E6 and E7 antigens by VGX-3100. Molecular Therapy-Oncolytics. 2016;3.

126. Kenter GG, Welters MJ, Valentijn ARP, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. New England Journal of Medicine. 2009;361(19):1838-47.

127. van Poelgeest MI, Welters MJ, Vermeij R, Stynenbosch LF, Loof NM, Berends-van der Meer DM, et al. Vaccination against oncoproteins of HPV16 for noninvasive vulvar/vaginal lesions: lesion clearance is related to the strength of the T-cell response. Clinical Cancer Research. 2016;22(10):2342-50.

128. Welters MJ, Kenter GG, van Steenwijk PJdV, Löwik MJ, Berends-van der Meer DM, Essahsah F, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proceedings of the National Academy of Sciences. 2010;107(26):11895-9.

129. Ferguson A, Moore M, Fox H. Expression of MHC products and leucocyte differentiation antigens in gynaecological neoplasms: an immunohistological analysis of the tumour cells and infiltrating leucocytes. British journal of cancer. 1985;52(4):551.

130. Dietl J, Horny H-P, Buchholz F. Lymphoreticular cells in invasive carcinoma of the uterine cervix: an immunohistological study. International Journal of Gynecology & Obstetrics. 1991;34(2):179-82.

131. Ghosh AK, Moore M. Tumour-infiltrating lymphocytes in cervical carcinoma. European Journal of Cancer. 1992;28(11):1910-6.

132. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer research. 2004;64(15):5449-55.

133. van Steenwijk PJdV, Piersma SJ, Welters MJ, van der Hulst JM, Fleuren G, Hellebrekers BW, et al. Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16-specific T-cell response. Clinical Cancer Research. 2008;14(22):7188-95.

134. Rosenthal DW, DeVoti JA, Steinberg BM, Abramson AL, Bonagura VR. TH2-like chemokine patterns correlate with disease severity in patients with recurrent respiratory papillomatosis. Molecular Medicine. 2012;18(1):1338.

135. Feng Q, Wei H, Morihara J, Stern J, Yu M, Kiviat N, et al. Th2 type inflammation promotes the gradual progression of HPV-infected cervical cells to cervical carcinoma. Gynecologic oncology. 2012;127(2):412-9.

136. Fakhry C, Gillison ML. Clinical implications of human papillomavirus in head and neck cancers. Journal of Clinical Oncology. 2006;24(17):2606-11.

137. Chung CH, Gillison ML. Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. Clinical cancer research. 2009;15(22):6758-62.

138. Wansom D, Light E, Worden F, Prince M, Urba S, Chepeha DB, et al. Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. Archives of Otolaryngology–Head & Neck Surgery. 2010;136(12):1267-73.

139. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay NEH, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clinical cancer research. 2006;12(2):465-72.

140. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, et al. PD-1–expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. Cancer research. 2013;73(1):128-38.

141. Piersma SJ, Jordanova ES, van Poelgeest MI, Kwappenberg KM, van der Hulst JM, Drijfhout JW, et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. Cancer research. 2007;67(1):354-61.

142. Jordanova ES, Gorter A, Ayachi O, Prins F, Durrant LG, Kenter GG, et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? Clinical Cancer Research. 2008;14(7):2028-35.

143. Wolf GT, Schmaltz S, Hudson J, Robson H, Stackhouse T, Peterson KA, et al. Alterations in T-lymphocyte subpopulations in patients with head and neck cancer: correlations with prognosis. Archives of Otolaryngology–Head & Neck Surgery. 1987;113(11):1200-6.

144. Heusinkveld M, Welters MJ, van Poelgeest MI, van der Hulst JM, Melief CJ, Fleuren GJJ, et al. The detection of circulating human papillomavirus-specific T cells is associated with improved survival of patients with deeply infiltrating tumors. International journal of cancer. 2011;128(2):379-89.

145. Khazaie K, Von Boehmer H, editors. The impact of CD4+ CD25+ Treg on tumor specific CD8+ T cell cytotoxicity and cancer. Seminars in cancer biology; 2006: Elsevier.

146. Piersma SJ, Welters MJ, van der Burg SH. Tumor-specific regulatory T cells in cancer patients. Human immunology. 2008;69(4-5):241-9.

147. Van der Burg SH, Piersma SJ, de Jong A, van der Hulst JM, Kwappenberg KM, van den Hende M, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. Proceedings of the National Academy of Sciences. 2007;104(29):12087-92.

148. Visser J, Nijman H, Hoogenboom BN, Jager P, Van Baarle D, Schuuring E, et al. Frequencies and role of regulatory T cells in patients with (pre) malignant cervical neoplasia. Clinical & Experimental Immunology. 2007;150(2):199-209.

149. Peghini BC, Abdalla DR, Barcelos ACM, Teodoro LdGVL, Murta EFC, Michelin MA. Local cytokine profiles of patients with cervical intraepithelial and invasive neoplasia. Human immunology. 2012;73(9):920-6.

150. Ali KS, Ali HY, Jubrael JM. Concentration levels of IL-10 and TNF α cytokines in patients with human papilloma virus (HPV) DNA+ and DNA− cervical lesions. Journal of immunotoxicology. 2012;9(2):168-72.

151. Bermúdez-Morales VH, Gutiérrez LX, Alcocer-González JM, Burguete A, Madrid-Marina V. Correlation between IL-10 gene expression and HPV infection in cervical cancer: a mechanism for immune response escape. Cancer investigation. 2008;26(10):1037-43.

152. Bolpetti A, Silva JS, Villa LL, Lepique AP. Interleukin-10 production by tumor infiltrating macrophages plays a role in Human Papillomavirus 16 tumor growth. BMC immunology. 2010;11(1):27.

153. Welters MJ, van der Sluis TC, van Meir H, Loof NM, van Ham VJ, van Duikeren S, et al. Vaccination during myeloid cell depletion by cancer chemotherapy fosters robust T cell responses. Science translational medicine. 2016;8(334):334ra52-ra52.

154. Heeren AM, de Boer E, Bleeker MC, Musters RJ, Buist MR, Kenter GG, et al. Nodal metastasis in cervical cancer occurs in clearly delineated fields of immune suppression in the pelvic lymph catchment area. Oncotarget. 2015;6(32):32484.

155. Heeren AM, Koster BD, Samuels S, Ferns DM, Chondronasiou D, Kenter GG, et al. High and interrelated rates of PD-L1+ CD14+ antigen-presenting cells and regulatory T cells mark the microenvironment of metastatic lymph nodes from patients with cervical cancer. Cancer immunology research. 2014:canimm. 0149.2014.

156. Pahne-Zeppenfeld J, Schröer N, Walch-Rückheim B, Oldak M, Gorter A, Hegde S, et al. Cervical cancer cell-derived interleukin-6 impairs CCR7-dependent migration of MMP-9-expressing dendritic cells. International journal of cancer. 2014;134(9):2061-73.

157. Matthews K, Leong CM, Baxter L, Inglis E, Yun K, Bäckström BT, et al. Depletion of Langerhans cells in human papillomavirus type 16-infected skin is associated with E6-mediated down regulation of E-cadherin. Journal of virology. 2003;77(15):8378-85.

158. Iijima N, Goodwin EC, DiMaio D, Iwasaki A. High-risk human papillomavirus E6 inhibits monocyte differentiation to Langerhans cells. Virology. 2013;444(1-2):257-62.

159. Heusinkveld M, van Steenwijk PJdV, Goedemans R, Ramwadhdoebe TH, Gorter A, Welters MJ, et al. M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. The Journal of Immunology. 2011;187(3):1157-65.

160. De Vos van Steenwijk P, Ramwadhdoebe T, Goedemans R, Doorduijn E, Van Ham J, Gorter A, et al. Tumor-infiltrating CD14-positive myeloid cells and CD8-positive T-cells prolong survival in patients with cervical carcinoma. International journal of cancer. 2013;133(12):2884-94.

161. van der Sluis TC, Sluijter M, van Duikeren S, West BL, Melief CJ, Arens R, et al. Therapeutic peptide vaccine-induced CD8 T cells strongly modulate intratumoral macrophages required for tumor regression. Cancer immunology research. 2015;3(9):1042-51.

162. Regad T. Targeting RTK signaling pathways in cancer. Cancers. 2015;7(3):1758-84.

163. Henson ES, Gibson SB. Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. Cellular signalling. 2006;18(12):2089-97.

164. Lizcano JM, Alessi DR. The insulin signalling pathway. Current biology. 2002;12(7):R236-R8.

165. Asnaghi L, Bruno P, Priulla M, Nicolin A. mTOR: a protein kinase switching between life and death. Pharmacological research. 2004;50(6):545-9.

166. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nature reviews Molecular cell biology. 2001;2(2):127.

167. Walker F, Abramowitz L, Benabderrahmane D, Duval X, Descatoire V, Hénin D, et al. Growth factor receptor expression in anal squamous lesions: modifications associated with oncogenic human papillomavirus and human immunodeficiency virus. Human pathology. 2009;40(11):1517-27.

168. Charpidou A, Blatza D, Anagnostou E, Syrigos KN. EGFR mutations in non-small cell lung cancer-clinical implications. In vivo. 2008;22(4):529-36.

169. Ooi A, Takehana T, Li X, Suzuki S, Kunitomo K, Iino H, et al. Protein overexpression and gene amplification of HER-2 and EGFR in colorectal cancers: an immunohistochemical and fluorescent in situ hybridization study. Modern pathology. 2004;17(8):895.

170. Hanawa M, Suzuki S, Dobashi Y, Yamane T, Kono K, Enomoto N, et al. EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. International journal of cancer. 2006;118(5):1173-80.

171. Lin G, Sun XJ, Han QB, Wang Z, Xu YP, Gu JL, et al. Epidermal growth factor receptor protein overexpression and gene amplification are associated with aggressive biological behaviors of esophageal squamous cell carcinoma. Oncology letters. 2015;10(2):901-6.

172. Gan HK, Cvrljevic AN, Johns TG. The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered. The FEBS journal. 2013;280(21):5350-70.

173. Sasada T, Azuma K, Ohtake J, Fujimoto Y. Immune responses to epidermal growth factor receptor (EGFR) and their application for cancer treatment. Frontiers in pharmacology. 2016;7:405.

174. Azuma K, Ota K, Kawahara A, Hattori S, Iwama E, Harada T, et al. Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. Annals of oncology. 2014;25(10):1935-40.

175. Chen N, Fang W, Zhan J, Hong S, Tang Y, Kang S, et al. Upregulation of PD-L1 by EGFR activation mediates the immune escape in EGFR-driven NSCLC: implication for optional immune targeted therapy for NSCLC patients with EGFR mutation. Journal of Thoracic Oncology. 2015;10(6):910-23.

176. Ota K, Azuma K, Kawahara A, Hattori S, Iwama E, Tanizaki J, et al. Induction of PD-L1 expression by the EML4–ALK oncoprotein and downstream signaling pathways in non–small cell lung cancer. Clinical cancer research. 2015;21(17):4014-21.

177. Akbay EA, Koyama S, Carretero J, Altabef A, Tchaicha JH, Christensen CL, et al. Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. Cancer discovery. 2013;3(12):1355-63.

178. Concha-Benavente F, Srivastava RM, Ferrone S, Ferris RL. EGFR-mediated tumor immunoescape: the imbalance between phosphorylated STAT1 and phosphorylated STAT3. Oncoimmunology. 2013;2(12):e27215.

179. Seshacharyulu P, Ponnusamy MP, Haridas D, Jain M, Ganti AK, Batra SK. Targeting the EGFR signaling pathway in cancer therapy. Expert opinion on therapeutic targets. 2012;16(1):15-31.

180. Trivedi S, Srivastava RM, Concha-Benavente F, Ferrone S, Garcia-Bates TM, Li J, et al. Anti-EGFR targeted monoclonal antibody isotype influences antitumor cellular immunity in head and neck cancer patients. Clinical Cancer Research. 2016;22(21):5229-37.

Chapter 2

Human papillomavirus downregulates the expression of IFITM1 and RIPK3 to escape from IFNγ-and TNFα-mediated anti-proliferative effects and necroptosis

Ma W, Tummers B, Van Esch EM, Goedemans R, Melief CJ, Meyers C, Boer JM, Van Der Burg SH.

Front Immunol. 2016 Nov 22;7:496

Human papillomavirus (HPV) downregulates the expression of IFITM1 and RIPK3 to escape from IFNγ and TNFα-mediated anti-proliferative effects and necroptosis

Wenbo Ma[1*], Bart Tummers[1*], Edith M.G. van Esch[2], Renske Goedemans[1], Cornelis J.M. Melief[3], Craig Meyers[4], Judith M. Boer[5], Sjoerd H. van der Burg[1,§].

[1]Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands.
[2]Department of Gynaecology, Leiden University Medical Center, Leiden, The Netherlands.
[3]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands.
[4]Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Hershey, USA
[5]Human Genetics, Leiden University Medical Center, Leiden, The Netherlands; Current address: Department of Pediatric Oncology, Erasmus MC - Sophia Children's Hospital, Rotterdam, The Netherlands and Netherlands Bioinformatics Center, Nijmegen, The Netherlands

* Both authors contributed equally

[§]Corresponding author: Prof. Dr. S.H. van der Burg, Dept. of Clinical Oncology, Building 1, K1-P, Leiden University Medical Center, PO box 9600, 2300 RC Leiden, The Netherlands. Phone: +31-71-5261180, Fax: +31-71-5266760, E-mail: shvdburg@lumc.nl

Key words
HPV, infection, immune escape, Th1 cytokines.

Abstract
The clearance of a high-risk human papillomavirus (hrHPV) infection takes time and requires the local presence of a strong type 1 cytokine T cell response, suggesting that hrHPV has evolved mechanisms to resist this immune attack. Using an unique system for non, newly and persistent hrHPV infection, we show that hrHPV infection renders

keratinocytes (KCs) resistant to the anti-proliferative and necroptosis inducing effects of IFNγ and TNFα. HrHPV-impaired necroptosis was associated with the upregulation of several methyltransferases, including EZH2 and the downregulation of RIPK3 expression. Restoration of RIPK3 expression using the global histone methyltransferase inhibitor 3-deazaneplanocin increased necroptosis in hrHPV-positive KCs. Simultaneously, hrHPV effectively inhibited IFNγ/TNFα-mediated arrest of cell growth at the S-phase by downregulating *IFITM1* already at 48 hours after hrHPV infection, followed by an impaired increase in the expression of the anti-proliferative gene *RARRES1* and a decrease of the proliferative gene *PCNA*. Knockdown of *IFITM1* in uninfected KCs confirmed its role on *RARRES1* and its anti-proliferative effects. Thus, our study reveals how hrHPV deregulates two pathways involved in cell death and growth regulation to withstand immune mediated control of hrHPV-infected cells.

Introduction

High-risk human papillomaviruses (hrHPVs) infect undifferentiated keratinocytes (KCs) of squamous epithelia. Persistent infections may lead to cancers of the anogenital region as well as of the head and neck [1]. In order to establish a persistent and productive infection, hrHPV requires access to the undifferentiated KCs that make up the epithelial basal layer and have the capacity to divide [2]. High-risk HPV infections can persist despite viral activity in keratinocytes, indicating that HPV has developed mechanisms to evade or suppress the innate and/or adaptive immune response of the host. Indeed, hrHPV utilizes its viral proteins and exploits cellular proteins to interfere with signaling of innate immune pathways, potentially postponing the activation of an adaptive immune response [3]. HPV may attenuate immune signaling at different levels in the STAT [4-7], IRF and NFκB pathways [8-14], and has also been shown to impair IFNγ and TNFα signaling [15]. Nevertheless, T cells will become activated and migrate to infected sites. Studies in healthy individuals, immunosuppressed patients and in patients with spontaneously or vaccine-induced

regressions revealed an important role for a strong type 1 (IFNγ and TNFα)-associated HPV early antigen-specific T cell response in the control of HPV infected lesions [16]. However, even vaccines that boost viral Th1 immunity during chronic infection are only partially successful [17-19] with a positive clinical outcome only in patients with a very strong Th1 response [18, 20], suggesting that hrHPV may have found ways to resist the effector cytokines of the adaptive immune system.

IFNγ is a pleiotropic cytokine that affects immune regulation, immune surveillance, inflammation, tumor suppression, and has antiviral as well as anti-proliferative properties. Binding of IFNγ to the IFNγ receptor (IFNγR) leads to JAK1/2-mediated STAT1 phosphorylation, dimerization and nuclear translocation that results in interferon-stimulated gene (ISG)-expression [21]. IFNγ may also induce necroptotic cell death by the JAK1/STAT1-dependent activation of proteins, encoded by interferon stimulated genes, that drive various aspects of the RIPK1–RIPK3 necrosome complex assembly [22], including the RNA-responsive protein kinase PKR which then interacts with RIPK1 to trigger necroptosis [23]. TNFα also regulates cell survival, apoptosis or necroptosis via an intricate network of signals that operate downstream of TNF receptor 1 (TNFR1). Binding of TNF to TNFR1 leads to NFκB activation through cIAP-mediated ubiquitylation of RIPK1. Under circumstances that RIPK1 is deubiquitylated it can associate with FADD to recruit and homodimerize caspase-8 leading to the induction of apoptosis. RIPK1 can also bind and activate RIPK3 after which MLKL is activated and necroptosis ensues. $cFLIP_L$ is expressed upon NFκB signaling (as *CFLAR*), heterodimerizes with caspase-8 on FADD and as such prevents apoptosis by abrogating full caspase-8 activation and necroptosis by disrupting the interaction between RIPK1 and RIPK3. [24].
IFNγ and TNFα are known to synergize in the suppression of KC proliferation [25]. IFNγ induces growth arrest and differentiation [26, 27]. TNFα also induces growth arrest but there are conflicting data concerning its capacity to induce cell death of primary KCs [25, 28].

Exposure of KCs to both IFNγ and TNFα potently stimulates the production of nitric oxide synthase (iNOS) to induce the upregulation of FasL, Fas receptor activation and subsequent caspase-mediated apoptosis of KCs [29], an effect to which differentiated KCs are especially sensitive [30]. However, the ability of HPV-infected undifferentiated KCs to resist the effects of IFNγ and/or TNFα on proliferation as well as the underlying mechanisms are not well understood. In this study, we evaluated the influence of hrHPV on the IFNγ and TNFα-mediated cell growth inhibition and cell death induction of undifferentiated KCs by functional and biochemical assays. We utilized a system that resembles the natural infection with HPV as closely as possible, comprising of primary KCs that stably maintain the hrHPV genome as episomes and were shown to undergo the entire differentiation-dependent HPV life cycle in organotypic raft cultures, non-infected primary KC cultures and primary KCs newly infected with authentic HPV16 virions (Karim et al., 2013), to show that hrHPV presence renders KCs more resistant to both necroptosis and the anti-proliferative effects instigated by IFNγ and TNFα, and reveal the biological mechanisms responsible.

Material and Methods

Ethics statement

The use of discarded human foreskin, cervical and vaginal KC 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 and HPV16- or 18-positive cervical neoplasia) used for staining. All sections and cell lines were derived from discarded tissues and de-identified, therefore no informed consent was necessary.

Cell culture

Primary cultures of human epithelial keratinocytes (KCs) were established from foreskin, vaginal, vulva and cervical tissues as previously described [31] and grown in keratinocyte serum-free medium (EpiLife® Medium, with 60 µM calcium supplemented with HKGS kit, Invitrogen, Breda, The Netherlands). KCs stably maintaining the full episomal HPV genome following electroporation (HPV-positive KCs) were grown in monolayer culture using E medium in the presence of mitomycin C (Sigma-Aldrich) treated J2 3T3 feeder cells [32, 33] for two passages and were then adapted to EpiLife® Medium for one passage before experimentation. J2 3T3 mouse fibroblasts were cultured in Iscove's modified Dulbecco's medium supplemented with 8% fetal bovine serum, 2 mM l-glutamine and 1% penicillin-streptomycin (complete IMDM medium) (Gibco-BRL, Invitrogen).

Reagents

Recombinant human TNFα (Rhtnf-a, Invivogen/bioconnect, France), Recombinant Human Interferonγ (11343536, Immunotools, Germany), BV-6 (S7597, Selleckchem, Netherlands), Pan Caspase Inhibitor Z-VAD-FMK (FMK001, R&Dsystems, USA), 3-Deazaneplanocin A (DZNep) hydrochloride (A8182, Apexbt, Netherlands), Nec-1s (2263-1, Biovision, California, USA), Cycloheximide (CHX) solution (C4859, SIGMA, Netherlands), GSK503 (S7804, Selleckchem, Netherlands).

Analysis of IFNGR1, TNFR1 and TNFR2 cell surface expression

The expression of the receptors for the cytokines IFNγ and TNFα was analyzed by flow cytometry after staining of the cells with the antibodies (1:10 diluted) against IFNGRα (Mouse anti-human CD119-PE, clone GIR-94, BD Biosciences, Breda, the Netherlands), TNFR1 (mouse anti-human CD120a-PE, clone 16803, R&D systems, Abingdon, UK) or TNFR2 (mouse anti-human CD120b-PE, clone MR2.1, Invitrogen Life Technologies, Bleiswijk, the Netherlands). Briefly, cells were transferred into wells of V-bottom 96-wells plate, washed with ice-cold PBS+0.5% BSA and incubated for 10 minutes on ice with ice-cold PBS (B.Braun, Melsungen, Germany)+0.5% Bovine Serum Albumin (Sigma Aldrich) +10% Fetal Calf Serum(PAA Laboratories, Lelystad, the Netherlands). Then, the cells were washed again and

incubated for 30 minutes on ice in the dark with the antibody indicated. Following one wash step the cells were fixed in 1% paraformaldehyde before they were acquired by BD Fortessa with BD FACSDiv software version 6.2, and data analyzed using FlowJo version 10.0.7 (Treestar, Olten, Switzerland).

HPV16 knock-down in HPV16-positive KCs and infection of undifferentiated keratinocytes

HPV16-positive KCs were transfected with 50 nM Control or HPV16 E2 siRNA as previously described [13]. Primary basal layer human foreskin keratinocytes were infected with native HPV16 at MOI 100 as previously described [13]. Cells were washed and harvested and target gene expression was assayed by RT-qPCR.

IFITM1 knock-down in undifferentiated KCs

shRNA's were obtained from the MISSION TRC-library of Sigma-Aldrich (Zwijndrecht, The Netherlands). The MISSION shRNA clones are sequence-verified shRNA lentiviral plasmids (pLKO.1-puro) provided as frozen bacterial glycerol stocks (Luria Broth, carbenicillin at 100 µg/ml and 10% glycerol) in E. coli for propagation and downstream purification of the shRNA clones. pLKO.1 contains the puromycin selection marker for transient or stable transfection. The construct against IFITM1 (NM_003641) was TRCN0000057499: CCGGCCTCATGACCATTGGATTCATCTCGAGATGAATCCAATGGTCATGA GGTTTTTG and the control was: SHC004 (MISSION TRC2-pLKO puro TurboGFP shRNA Control vector):CCGGCGTGATCTTCACCGACAAGATCTCGAGATCTTGTCGGTGAA GATCACGT TTTT. KCs at ~60% confluency were transduced with lentivirus at MOI 5-10 over night, after which medium was replaced. At least 72 hours post-transduction cells were harvested, washed and plated as indicated and allowed to attach overnight. Cell were stimulated as indicated and assayed accordingly.

RNA expression analyses

The micro array data [14] is accessible in the Gene Expression Omnibus database (accession number GSE54181). Plots were

generated using the webtool R2: microarray analysis and visualization platform (http://r2.amc.nl).
Total RNA was isolated using the NucleoSpin RNA II kit (Machery-Nagel, Leiden, The Netherlands) according to the manufacturer's instructions. Total RNA (0.5 – 1.0 µg) was reverse transcribed using the SuperScript III First Strand synthesis system from Invitrogen. TaqMan PCR was performed using TaqMan Universal PCR Master Mix and pre-designed, pre-optimized primers and probe mix for IFITM1, GLB1, BCL-2, Bax, RARRES1, PCNA and GAPDH (Applied Biosystems, Foster City, USA). Threshold cycle numbers (Ct) were determined using the CFX PCR System (BioRad, Veenendaal, The Netherlands) and the relative quantities of cDNA per sample were calculated using the ΔΔCt method using GAPDH as the calibrator gene.

Western blot analysis

Polypeptides were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Veenendaal, The Netherlands). Immunodetection was achieved with primary antibodies against TRAF2 (#4724s, Cell Signaling Technology (CST), Leiden, Netherlands), cIAP1 (#7065p, CST), cIAP2 (#3130s,CST), XIAP (#14334, CST), RIPK1 (#3493, CST), cFLIP (#3210, CST), caspase-8 (#9746, CST), cleaved caspase-8 (#9496s, CST), FADD (#2782, CST), RIPK3 (#13526, CST), MLKL (#14993s, CST), phospho-MLKL (phospho S358; ab187091, Abcam, Cambridge, UK), EZH2 (612667, BD Biosciences, The Netherlands), IFITM1 (PA5-20989, Thermo Scientific, Netherlands), Trimethyl-Histone H3 (Lys27)(07-449, Merk Millipore), STAT1 (#9172, CST), phospho-STAT1 (Tyr701, #9167, CST), β-actin (A5316, Sigma-Aldrich, Germany), and HRP-coupled anti-mouse (#7076s, CST) and HRP-coupled anti-rabbit (#7074s, CST) secondary antibodies. Chemoluminescence reagent (#170-5060, Bio-Rad, Germany) was used as substrate and signal was scanned using the Chemidoc and accompanying Software (Image Lab Software Version 5.2.1, Bio-Rad).

Proliferation assay

KCs, hrHPV+KCs, control shRNA-expressing KCs, or IFITM1 shRNA-expressing KCs were seeded 5000 cell/well in 96-well plates and allowed to attach overnight. Cells were cultured in presence of indicated concentrations of IFNγ and/or TNFα in 150 µl for 96 hours. 15 µl/well MTT (3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl-2*H*-tetrazolum bromide) stock solution (5 mg/ml in 0.1 M PBS) was added for 3 hours. When the purple formazan precipitate was clearly visible under the microscope, bright light pictures were made using an Olympus IX51 inverse fluorescence microscope (Olympus, Zoeterwoude, The Netherlands). Images were captured by ColorView II Peltier-cooled charge-coupled device camera (Olympus) and archived using Cell^F software (Olympus).

Cellular DNA content analysis.

The CyQuant-NF assay (C35006, ThermoFisher Scientific, USA), which measures cellular DNA content via fluorescent dye binding, was used to quantify the cell number in cultures treated with increasing doses of IFNγ. Briefly, control shRNA-expressing KCs, or IFITM1 shRNA-expressing KCs were seeded 500 cell/well in 96-well plates and allowed to attach overnight. Cells were cultured in presence of indicated concentrations of IFNγ, in triplicate wells, for 96 hours and then processed according to the protocol for adherent cells provided by the manufacturer. The fluorescence intensity detected is a measure for the number of cells present in the wells.[34]

Cell cycle analysis of keratinocytes

Following the treatment of KCs with 250 IU/ml IFNγ for 48 hours, the cells were fixed in 70% ethanol at 4˚C overnight. The fixed cells were washed with cold PBS and subsequently incubated for 30 minutes with 10 µg/ml RNase (#R6513, Sigma-aldrich) and 10 µg/ml propidium iodide (P4170, Sigma-Aldrich) staining. Cell cycle was detected by flow cytometry (BD Accuri™ C6, BD biosciences, The Netherlands) and analyzed using FlowJo v10.0.8.

SYTOX green dead cell assay

Replicate cultures of cells were plated in 6-wells tissue culture plates. Following the indicated treatments, all adhering and floating cells were collected with TrypLE™ Express Enzyme (12604-021, Thermofisher). The cells were washed with HBSS (14025-092, Thermofisher) once and then incubated with 5 µM SYTOX® Green Nucleic Acid Stain (S7020, Thermofisher) in absence or presence of 1µg/ml DAPI (D9542, Sigma Aldrich) at 20°C for 30 min. The cells were washed with HBSS and mixed with VECTASHIELD Antifade Mounting Medium (Vectorlabs H-1000). 30 µl cell suspension was added to a glass section slide and examined by fluorescence microscopy. Non-DAPI stained cells were detected by flow cytometry (BD Accuri™ C6) and analyzed using FlowJo v10.0.8.

Statistics

Statistical analysis was performed using GraphPad Prism version 6.02. P values were determined using Welch-corrected unpaired t-tests or one-way Anova Tukey's multiple comparisons test. Ns: no significance. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Results

HPV-infected KCs have an altered expression of genes related to necroptosis and proliferation in response to IFNγ and TNFα stimulation.

We previously reported our validated microarray in which four independent uninfected KC and four independent hrHPV-infected KC cultures were stimulated with control or IFNγ [14, 31]. Analysis of marker genes in this array for necroptosis (*RIPK3, MLKL*), proliferation (*RARRES1, PCNA*), intrinsic apoptosis (*BCL-2, BAX*), extrinsic apoptosis (*FADD, CFLAR*) and senescence (*GLB1, DEP1*) revealed that hrHPV-infection was specifically associated with changes in the genes associated with necroptosis and proliferation (Figure 1A). *RIPK3*, a crucial regulator of necroptosis, and its downstream partner *MLKL* [35] were both down-regulated in IFNγ-stimulated hrHPV-infected KCs (Figure 1A). Furthermore, the increase in *RARRES1*, a marker for anti-proliferation [36, 37], and the decrease in *PCNA*, a marker of proliferation, observed in KCs treated with IFNγ for 24 hours was not seen in hrHPV-positive KCs where expression of these genes remained almost unaltered (Figure 1A). These data suggest that hrHPV-infected KCs can resist the growth regulatory effects of IFNγ and/or the combination of IFNγ and TNFα. Uninfected KCs and hrHPV-infected KCs express the IFNγ receptor 1 (IFNGR1), and the TNFα receptors 1 and 2 (TNFR1 and TNFR2) at the cell surface enabling them to respond to these cytokines (Supplemental Figure 1A). When these cells were seeded into 96-well plates and treated for four days with increasing doses of IFNγ and/or TNFα, the growth of uninfected KCs was greatly affected by IFNγ in a dose-dependent manner while hrHPV-positive KCs were much more resistant to growth inhibition and still able to expand to a confluent cell layer (Supplemental Figure 1B). TNFα in itself appeared not to affect the growth of uninfected or HPV-infected KCs, but when combined with IFNγ it exaggerated the reduction in cell density (Supplemental Figure 1B). To confirm these results, KCs were harvested after IFNγ/TNFα stimulation, and the gene expression of the previously indicated markers indicative for proliferation, senescence, apoptosis and necroptosis were determined by RT-qPCR (Figure 1B). The qPCR showed that *RIPK3* is lower in hrHPV-infected KCs both at the basal level and after

treatment, while the effect on *MLKL* was less pronounced. Moreover, the increase in *RARRES1* and downregulation of *PCNA* was much lower in treated hrHPV-infected KC than in non-infected KCs (**Figure 1B**). Similar to the microarray, the marker genes for intrinsic and extrinsic apoptosis as well as senescence did not overtly differ between KCs and hrHPV-positive KCs. Together, our results show that specifically the necroptosis associated gene *RIPK3* and the anti-proliferative gene *RARRES1* were expressed lower in hrHPV-positive KCs. This suggests that the maintained proliferation of hrHPV-infected undifferentiated KCs during IFNγ and/or TNFα treatments is associated with a resistance to cell death at the level of necroptosis and by resistance to proliferation arrest but less likely to be regulated at the level of senescence or apoptosis.

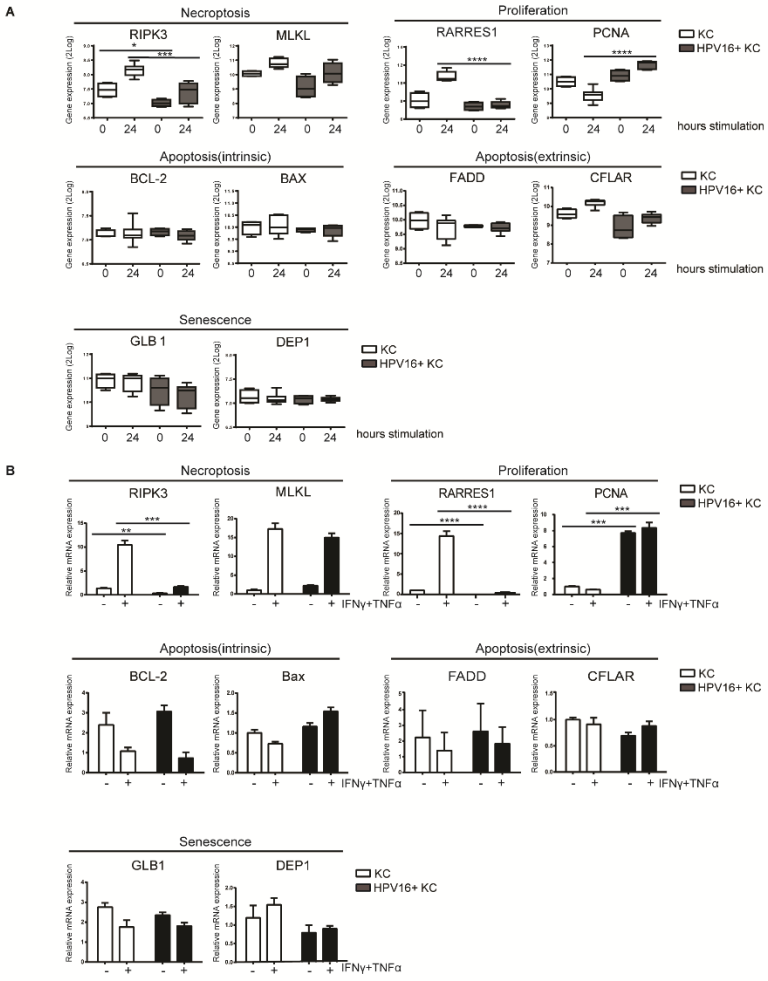


Figure 1. HPV-infected KCs display an altered expression of genes related to necroptosis and proliferation.

(A) Microarray gene expression values for *RIPK3, MLKL, RARRES1, PCNA, BCL-2, BAX, FADD, CFLAR, GLB1* and *DEP1* of 4 independent uninfected KCs and 4 independent hrHPV+ KCs, stimulated with IFNγ for 0 or 24 hours, represented in a box plot. The box represents the 25th and 75th percentiles, the median is indicated with a horizontal line within the box, and the whiskers represent the minimum and maximum. ***P<0.001 and ****P<0.0001.

(B) RT-qPCR *of RIPK3, MLKL, RARRES1, PCNA, BCL-2, BAX, FADD, CFLAR, GLB1* and *DEP1* in uninfected KCs and HPV16+KCs following treatment with 50 IU/ml IFNγ and 30 ng/ml TNFα for 24 hours. Gene expression was normalized against *GAPDH* mRNA levels and standardized against the non-stimulated uninfected KCs. Similar results were observed in two independent experiments. ****P<0.0001.

HPV suppresses necroptosis by downregulating RIPK3 expression.

Programmed cell death knows two major regulatory pathways: caspase-dependent apoptosis and RIP kinases-associated necroptosis [38]. To investigate the effects of hrHPV on these pathways, we analyzed the expression of the proteins TRAF2, cIAP1, cIAP2, XIAP, FLIP, CYLD, caspase-8, FADD, RIPK1, RIPK3, and MLKL, involved in formation of the apoptotic or necroptotic signaling complexes [38] at several different time points after IFNγ/TNFα stimulation (Figure 2). We found a consistent difference between KCs and hrHPV-positive KCs with respect to cIAP2, the expression of which was strongly upregulated at both the protein and transcript level by IFNγ/TNFα stimulation in hrHPV-positive KCs only (Figures 2A and 2D). The cytokine-stimulated expression of TRAF2 seemed to be higher in hrHPV-positive KCs but this was not confirmed at the transcript level (Figures 2A and 2D). Analysis of the apoptotic (FLIP, CYLD, caspase-8, FADD) or necroptotic (RIPK1, RIPK3, MLKL) proteins revealed that the levels of FADD were consistently upregulated in hrHPV-positive KCs in response to IFNγ/TNFα treatment. Again, this was not confirmed at the transcript level (Figures 2B and 2D). Similarly, there was a hint that cFLIP was upregulated at the protein level but not at the transcript level (Figures 2B and 2D). In addition, there was no difference between the different KCs in the expression caspase-8 or in the expression of partially cleaved caspase-8 (43 kD) after IFNγ/TNFα treatment (Figure 2B). Fully cleaved caspase-8 (18 kD and 10 kD) was not observed in these blots, unless KCs and hrHPV+KCs were treated with cyclohexamide, which fosters apoptosis by promoting full caspase-8 activation via the elimination of c-FLIP [39] (Supplemental Figure 2). Importantly, RIPK3 was downregulated significantly at the

protein level and transcript level by hrHPV (Figures 2C and 2D). Note that in hrHPV-positive KCs the increase in phosphorylated MLKL parallels that of MLKL itself and does not reflect a specific increase in MLKL phosphorylation (Figure 2C). Notably, the expression of many of the proteins in the apoptotic and necroptotic signaling complexes could be increased by either IFNγ or TNFα but most often the two cytokines synergized in raising the expression of these proteins, in particular RIPK3 (Supplemental Figure 3). The expression of the other components showed a similar expression in non-infected or hrHPV-positive KCs or varied between cell lines in a non-HPV related manner. To study the role of HPV in the consistently changed components cIAP2, FADD and RIPK3, the total HPV16 early gene expression was knocked down by introduction of siRNA against HPV16 E2 in hrHPV+KCs [13]. This resulted in the reduction of HPV16 gene expression in non- and IFNγ TNFα stimulated hrHPV+ KC (Figure 2E) as well as in the upregulation of *RIPK3* and unexpectedly also of *cIAP2,* while there was no change in *FADD* (Figure 2F). IFNγ TNFα stimulation augmented the expression of *cIAP2* and *RIPK3* in KCs when the polycistronic mRNA of HPV16 was knocked down (Figure 2F). These data indicate that only the altered expression of *RIPK3,* but not that of *cIAP2* and *FADD*, can be fully accounted for by an infection of KCs with hrHPV and suggest that hrHPV might impair IFNγ and TNFα induced necroptosis.

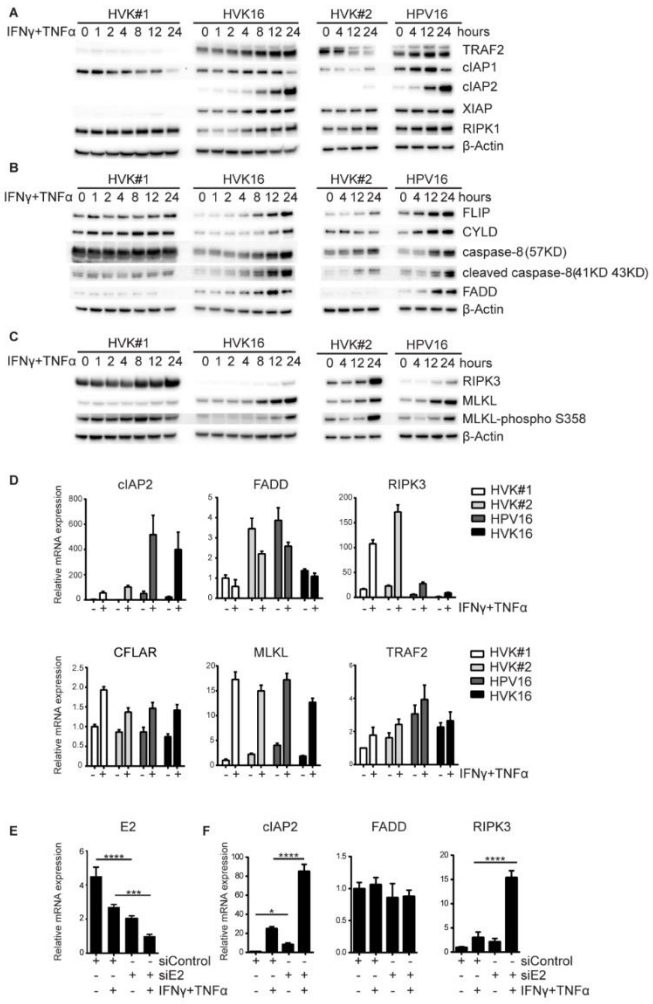


Figure 2. HPV suppresses the expression of RIPK3.

(A-C) Two independent uninfected KC cultures (HVK#1, HVK#2) and two HPV16+KC cultures (HVK16, HPV16) were stimulated with 50 IU/ml IFNγ and 30 ng/ml TNFα for the indicated times. The protein expression levels of (A) TRAF2, cIAP1, cIAP2, XIAP, RIP1 and (B) FLIP, CYLD, caspase-8 (57 kD), cleaved caspase-8 (41 kD, 43 kD), FADD, and (C) RIPK3, MLKL and MLKL-phospho S358 as detected by western blotting (WB) in whole cell extracts. β-actin served as loading control.

(D) Two independent uninfected KC cultures (HVK#1, HVK#2) and two HPV16+KC cultures (HVK16, HPV16) were stimulated with 50 IU/ml IFNγ and 30 ng/ml TNFα for 24 hours after which the expression levels of *cIAP2, FADD, RIPK3, CFLAR, MLKL* and *TRAF2* were determined by RT-qPCR. Gene expression was normalized against *GAPDH* mRNA levels and standardized against the non-stimulated HVK#1. Similar results were observed in two independent experiments.
(E) HPV16 *E2* expression in HPV+KCs transfected with control siRNA (siControl) or siRNA targeting HPV16 E2 (siE2) stimulated with or without 50 IU/ml IFNγ and 30 ng/ml TNFα for 24 hours. *E2* expression was analyzed by RT-qPCR. Gene expression was normalized against *GAPDH* mRNA levels and standardized against siControl. Similar results were observed in 3 independent experiments. ***P<0.001 and ****P<0.0001.
(F) Expression of *cIAP2, FADD* and *RIPK3* in hrHPV+ KCs transfected with control siRNA (siControl) or siRNA targeting HPV16 E2 (siE2) stimulated with or without 50 IU/ml IFNγ and 30 ng/ml TNFα for 24 hours. Gene expression was normalized against *GAPDH* mRNA levels and standardized against siControl. Similar results were observed in 2 independent experiments. *P<0.05, ****P<0.0001

To test this, KCs and hrHPV-positive KCs were stimulated for 48 hours with IFNγ and TNFα in the presence of the Smac mimetic BV6 and caspase inhibitor zVAD-fmk in order to promote necroptosis [40] and cell death was analyzed with SYTOX green dead cell stain. While about 80% of the non-infected KCs were killed, a significantly lower percentage, less than 40%, of the hrHPV+KCs died within this 48 hour time frame (Figure 3). Consistent with necroptosis, cell death was completely blocked when the RIP kinase 1 inhibitor necrostatin (Nec)-1s [41] was present during stimulation (Supplemental Figure 4). Together, these results show that hrHPV-infected KCs can escape from IFNγ/TNFα induced necroptosis by downregulating the basal and cytokine-induced expression of RIPK3.

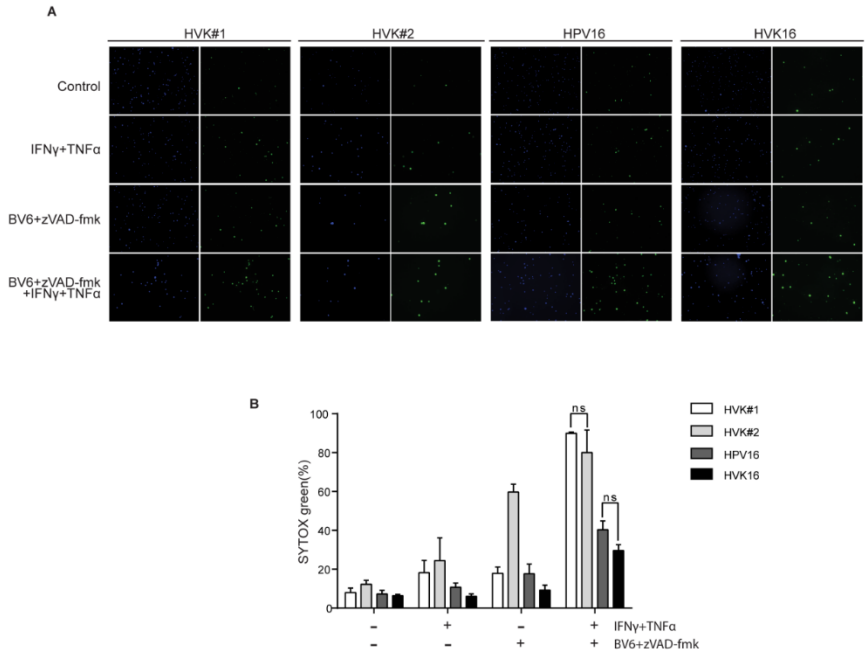


Figure 3. HPV increases the resistance of KCs to necroptosis.
(A) The two independent uninfected KC cultures (HVK#1, HVK#2) and two HPV16+ KC cultures (HVK16,HPV16) were stimulated with 250 IU/ml IFNγ, 250 ng/ml TNFα, 5 µM BV6, and 20 µM zVAD-fmk as indicated for 48 hours. All cell nuclei were labelled with DAPI (blue fluorescence). Dead cells were stained using SYTOX green dead cell stain resulting in green fluorescent nuclei of dead cells.
(B) The number of dead cells among all cells were counted in multiple fields. The percentage of cell death was calculated. P values were determined using one way anova Tukey's multiple comparisons test. In the group of IFNγ+TNFα+BV6+z-VAD-fmk, HVK#1 vs. HPV16, $P<0.01$; HVK#1 vs. HVK16, $P<0.001$; HVK#2 vs. HPV16, $P<0.05$; HVK#2 vs. HVK16, $P<0.05$

RIPK3 downregulation and resistance to necroptosis involves histone methyltransferases overexpressed in hrHPV-infected KCs

In many cancer cell lines the expression of RIPK3 is lost due to genomic methylation near its transcriptional start site [42]. Recently, it was reported that the methyltransferase EZH2 was overexpressed in HPV16 E6 and E7-transformed KCs [43]. Since EZH2 is a core component of polycomb repressive complex 2 (PRC2) and plays a role in promoter-targeted transcriptional repression [44], we hypothesized that EZH2 may also be involved in repressing the expression of *RIPK3*. Western blot analysis and RT-qPCR of primary KCs and hrHPV-infected KCs revealed that both gene and protein expression of EZH2 was higher at the basal level and after IFNγ/TNFα stimulation in hrHPV+KCs (Figures 4A and 4B). As expected [44], hrHPV-infected KCs display a higher methylation of H3K27 at the basal level (Supplemental Figure 5A). Knock-down of the polycistronic mRNA of HPV16 in hrHPV16-positive KCs resulted in lower EZH2 protein levels indicating that EZH2 overexpression was induced by hrHPV in KCs Figure 4C). This effect was even more pronounced after IFNγ/TNFα stimulation fitting with the observation that also the expression of the viral genes was further downregulated (Figure 2E). The use of 3-deazaneplanocin A (DZNep), an inhibitor of S-adenosylmethionine-dependent methyltransferase, is known to effectively deplete cellular levels of the PRC2 components, including EZH2 [45]. Indeed, treatment of the hrHPV+KCs with DZNep resulted in a dose-dependent decrease in EZH2 protein levels (Figure 4D), decreased of H3K27 methylation (Supplemental Figure 5B) and the concomitant increase in RIPK3 at the protein and gene expression level (Figures 4D and 4E). Treatment of hrHPV+KCs with the catalytic EZH2 inhibitor GSK503 did not have a clear effect on the expression of RIPK3 (Supplemental Figure 5C), suggesting that the hrHPV-induced overexpression of EZH2 is indirectly responsible for suppressing RIPK3 mediated necroptosis it should be noted that DZNep has been reported to function as a global histone methylation inhibitor [46]. We therefore analyzed the expression of other methyltransferases and found that, in addition to EZH2, 8 other methyltransferases were expressed at a significantly higher level in hrHPV+KCs (Supplemental Figure 5DE). Potentially, these methyltransferase may also play a role in downregulating the expression of RIPK3. To test if

methyltransferases where involved in suppressing RIPK3-mediated necroptosis, KCs and hrHPV+KCs again were stimulated with IFNγ/TNFα, BV6 and zVAD-fmk but now either in the absence or presence of DZNep. Cell death was determined both by flow cytometry in order to analyze larger numbers of cells (Figure 4F), and by immunofluorescence in cell cultures (Supplemental Figure 6). Clearly, the presence of DZNep increased the BV6/zVAD-fmk/IFNγ/TNFα-induced percentage of dead cells. Cells died by necroptosis, since cell death could be blocked by necrostatin (Nec)-1s (Figures 4F and 4G; Supplemental Figure 6).

Thus, by upregulating the expression of histone methyltransferases, thereby effectively decreasing the basal levels of RIPK3, hrHPV increases the immune resistance of KCs to IFNγ/TNFα stimulated necroptotic cell death.

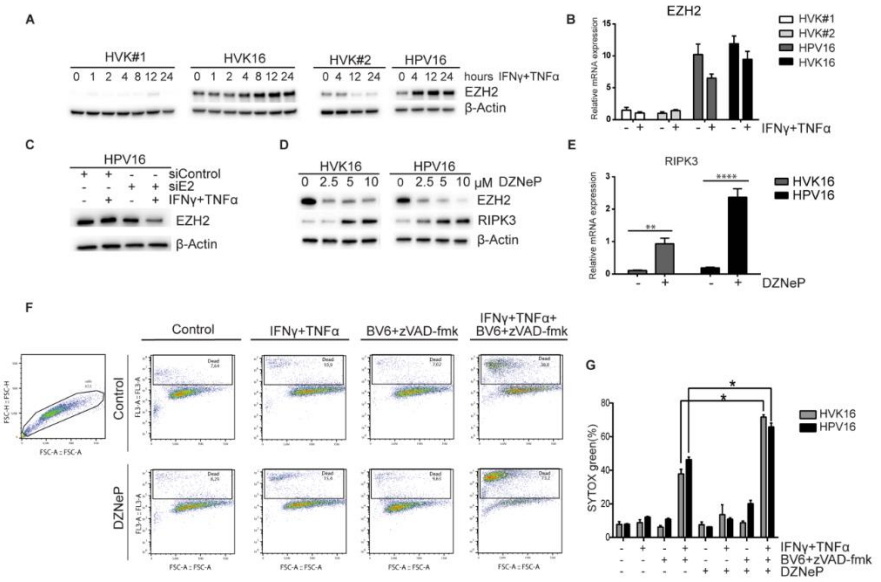


Figure 4. High risk HPV-infected KCs overexpress EZH2 and downregulates RIPK3 and to resist necroptosis.

(A) Two independent uninfected KC cultures (HVK#1, HVK#2) and two HPV16+KC cultures (HVK16, HPV16) were stimulated with 50 IU/ml IFNγ and 30 ng/ml TNFα for the indicated times following the protein

levels of EZH2 as detected by western blotting (WB) in whole cell extracts. β-actin served as loading control.
(B) Two independent uninfected KC cultures (HVK#1, HVK#2) and two HPV16+KC cultures (HVK16, HPV16) were stimulated with 50 IU/ml IFNγ and 30 ng/ml TNFα for 24 hours after which the expression levels of *EZH2* was determined by RT-qPCR. Gene expression was normalized against *GAPDH* mRNA levels and standardized against the non-stimulated uninfected KC culture HVK#1.
(C) The protein expression level of EZH2 was analyzed in HPV16+KCs (HPV16) transfected with control siRNA (siControl) or siRNA targeting HPV16 E2 (siE2) and stimulated with or without 50 IU/ml IFNγ and 30 ng/ml TNFα for 24 hours, using western blotting (WB) in whole cell extracts. β-actin served as loading control.
(D) The protein expression levels of EZH2 and RIPK3 were analyzed in the two independent hrHPV+KCs (HVK16, HPV16) either 72 hours after pharmacological depletion of EZH2 by increasing doses of 3-deazaneplanocin (DZNeP) .
(E) The gene expression level of *RIPK3* in 10μM DZNeP-treated HVK16 and HPV16 hrHPV+ KCs after 72 hours. Gene expression was normalized against *GAPDH* mRNA levels and standardized against the non-treated HVK16. **P<0.01, ****P<0.0001.
(F,G) Two hrHPV+ KCs (HVK16, HPV16) were treated with or without 10 μM DZNeP. After 24 hours, the cells were treated with 250 IU/ml IFNγ, 250 ng/ml TNFα, 5 μM BV6, 20 μM zVAD-fmk and/or 20 μM Nec-1s as indicated for 48 hours. The percentage of dead cells, indicated by the cells stained positive by SYTOX green dead cell stain, was measured by flow cytometry. (F) Example of analysis by flow cytometry. (G) The percentage of cell death measured for each treatment was plotted for both hrHPV+ KC cultures. **P < 0.05.

The anti-proliferative effects of IFNγ in KCs are counteracted by hrHPV through downregulation of interferon-induced transmembrane protein 1 (IFITM1).

Our initial analyses suggested that hrHPV-infected KCs resisted immune-controlled cell growth not only via impairment of necroptosis but also by interfering with regulation of proliferation,

exemplified by a lower increase in *RARRES1* and downregulation of *PCNA* (Figure 1). Indeed, stimulation of non-infected KCs and hrHPV+KCs with a high dose of IFNγ affects the growth of non-infected KCs but not hrHPV+KCs (Figure 5A). Furthermore, analysis of the cell cycle using flow cytometry and propidium iodide DNA staining, showed that treatment of KCs with IFNγ caused a 50% reduction in the S-phase while it hardly affected hrHPV+KCs (Figures 5B and 5C).

IFNγ signaling requires STAT1 and it has been reported that HPV can lower *STAT1* expression and protein levels in KCs [4-7], potentially explaining the resistance to IFNγ treatment. Indeed, hrHPV-positive KCs displayed a lower expression of STAT1 at the protein level (Figure 5D; Supplemental Figure 7), and this was due to the presence of hrHPV as knock-down of the polycistronic viral mRNA resulted in a higher *STAT1* expression (Figure 5E). Notably, cytokine stimulation also upregulated *STAT1* expression (Figure 5E), and IFNγ stimulation resulted in high levels of phosphorylated STAT1 in hrHPV+KCs (Figure 5D), indicating that HPV may repress the basal levels of STAT1 but it does not overtly interfere with STAT1 signaling *per se*. This also explains why IFNγ, especially at higher concentrations and in combination with TNFα, is able to stimulate the expression of the anti-proliferative gene *RARRES1* and downregulation of the proliferative gene *PCNA* (Figure 5F), albeit that the combinatory effects of IFNγ and TNFα were more pronounced on non-infected KCs (Figure 5F).

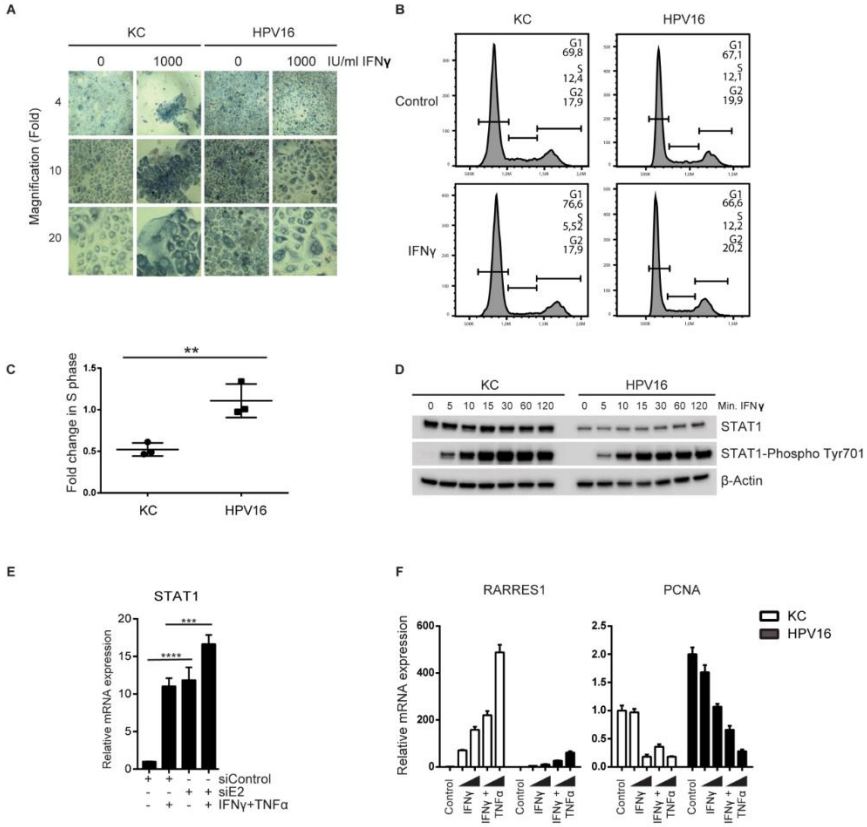


Figure 5. High-risk HPV resists the anti-proliferative effects of IFNγ.

(A) Undifferentiated KCs (HVK#1) and HPV16+KCs (HVK16) were treated with indicated doses of IFNγ for 72 hours after which cell confluency was monitored by phase-contrast microscopy as a measure of proliferation. Microscopy pictures (4x, 10x and 20x magnifications).

(B,C) The proliferation of undifferentiated KC (HVK#2) and HPV16+KC (HPV16) treated with 250 IU/ml IFNγ for 48 hours was analyzed by examination of the proportion of DNA that was present in the various

phases (G1, S, G2/M) of cell growth using PI staining and flow cytometry. (B) Example of flow cytometric analysis. The DNA content of 10,000 cells was analyzed. (C) The fold change in the percentage of cells in the S phase of treated cells over non-treated cells of n=3 experiments is shown. **P<0.01.
(D) The protein levels of STAT1 and phosphorylated STAT1 at Tyr701 protein levels in undifferentiated KCs and HPV16+KCs harvested at indicated time points after stimulation with 50 IU/ml IFNγ as measured by WB is shown. β-actin served as loading control.
(E) Expression of *STAT1* in HPV16+ KCs transfected with control siRNA (siControl) or siRNA targeting HPV16 E2 (siE2) stimulated with or without 50 IU/ml IFNγ and 30 ng/ml TNFα for 24 hours. Gene expression was normalized against *GAPDH* mRNA levels and fold-change over non-stimulated siControl was calculated. ****P<0.0001, ***P<0.001.
(F) Gene expression analysis of *RARRES1* and *PCNA* in undifferentiated KCs and HPV16+ KCs treated with 50 IU/ml IFNγ and 30 ng/ml TNFα for 24 hours. Gene expression was normalized against *GAPDH* mRNA levels and fold change over control was calculated.

IFITM1 plays an essential role in the anti-proliferative action of IFNγ [47], making it a potential target for hrHPV. Re-analysis of the data from one of our earlier validated microarrays, in which the basal expression of genes measured in different uninfected and hrHPV infected KCs was compared in the absence of IFNγ stimulation [31], showed that *IFITM1* expression is downregulated in HPV-positive KCs (Figure 6A). This was confirmed by RT-qPCR (Figure 6B). To show that the expression of IFITM1 was genuinely altered by the presence of hrHPV in KCs, undifferentiated KCs were infected with native HPV16 virions resulting in a reduced expression of *IFITM1* (Figure 6C). Reciprocally, the knock-down of total HPV16 early gene expression in hrHPV+KCs resulted in the upregulation of *IFITM1* (Figure 6D). IFNγ induces *de novo* synthesis of *IFITM1* for which STAT1 is required [48-51]. Indeed, IFNγ stimulation of uninfected KCs resulted in approximately 4-fold increase in *IFITM1* after 24 hours (Figure 6E). Strikingly, IFNγ stimulation of hrHPV+KCs resulted in a much stronger

relative increase of IFITM1 levels (Figure 6F), albeit that these levels still remained lower than those measured in uninfected KCs (Figure 6E). IFITM1 protein levels in IFNγ-stimulated KCs and hrHPV+KCs confirmed the gene expression data (Figure 6G). These data indicated that hrHPV predominantly regulates the expression of *IFITM1* at the basal level but less at the level of IFNγ-mediated induction of *IFITM1* gene expression. Interestingly, the hrHPV+KCs with the highest basal IFITM1 protein expression also showed the highest STAT1 levels (Supplemental Figure 7). TNFα did not influence *IFITM1* expression (Figures 6E and 6F).

To study the effects of IFITM1 on KC proliferation in a setting where all additional influences of HPV are ruled out [52, 53], IFITM1 was knocked-down in uninfected KCs (Figure 6H). The KCs were stimulated with IFNγ or IFNγ/TNFα. The basal level of *RARRES1* was lower in IFITM1 knocked-down KCs and its IFNγ-induced expression was clearly affected when KCs were stimulated with a low concentration of IFNγ (Figure 6I). A higher concentration of IFNγ overcame the effect of IFITM1 knock-down on the expression of *RARRES1*. In addition, IFITM1 knock-down KCs displayed a less strong downregulation of *PCNA* upon IFNγ stimulation (Figure 6I). The less pronounced effects on *RARRES1* and *PCNA* at the higher IFNγ concentrations used was probably due to the fact that upon IFNγ stimulation still an increase in *IFITM1* could be observed in IFITM1 knock-down KCs (Figure 6I). In addition, control shRNA-transduced KCs were less resistant than IFITM1 knock-down KCs to the anti-proliferative effects of IFNγ and the combination of IFNγ and TNFα when cell confluency was monitored by phase-contrast microscopy or cell number was quantitated by a DNA-based proliferation assay (Supplemental Figure 8AB). Thus, HPV is able to resist IFNγ-mediated arrest of proliferation by lowering the basal levels of IFITM1.

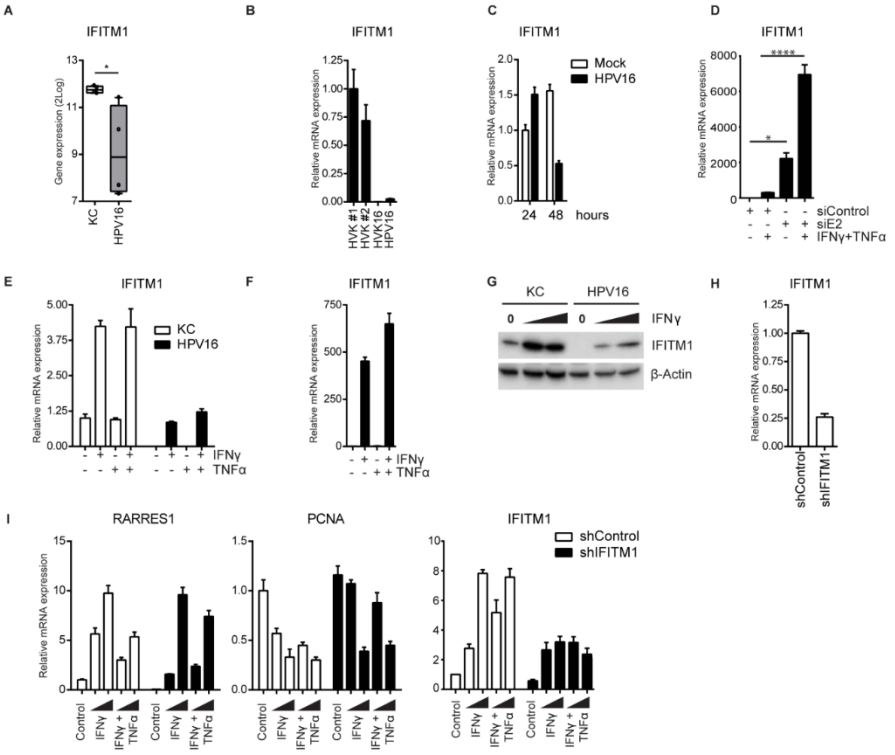


Figure 6. High-risk HPV-infected KCs downregulate IFITM1 to suppress the expression of *RARRES1* and to maintain proliferation.

(A) Microarray gene expression values for *IFITM1* in 4 independent uninfected KCs and 4 independent hrHPV+KCs represented in a box plot. * p<0.05.

(B) The expression level of *IFITM1* in HVK#1, HVK#2, HVK16 and HPV16 determined by RT-qPCR. Gene expression was normalized against *GAPDH* mRNA levels.

(C) The expression level of *IFITM1* in KCs infected with mock or native HPV16 virions for 1 or 2 days, respectively.

(D) Expression of *IFITM1* in HPV16+KCs transfected with control siRNA (siControl) or siRNA targeting HPV16 E2 (siE2) stimulated with or without 50 IU/ml IFNγ and 50 ng/ml TNFα for 24 hours. Gene expression was normalized against *GAPDH* mRNA levels and fold-change over non-stimulated siControl was calculated. *P<0.05, ****P<0.0001.

(E, F) The expression of *IFITM1* in undifferentiated KCs and HPV16+KCs stimulated with 50 IU/ml IFNγ and/or 50 ng/ml TNFα for 24 hours. Gene expression was normalized against *GAPDH* mRNA levels and fold changes over (E) control-stimulated undifferentiated KCs or over (F) control-stimulated HPV16+KCs were calculated and depicted.
(G) IFITM1 protein levels in KC and HPV16+KC stimulated with 0, 100 or 1000 IU/ml IFNγ, as measured by WB. β-actin served as loading control.
(H) *IFITM1* expression in control and IFITM1 knockdown uninfected KCs as measured by RT-qPCR. Gene expression was normalized against *GAPDH* mRNA levels and fold-change over siControl was calculated.
(I) Control and *IFITM1* knockdown KCs were stimulated with 50 IU/ml IFNγ and/or 50 ng/ml TNFα for 24 hours before the expression of *RARRES, PCNA, and IFITM1* was measured by RT-qPCR. Gene expression was normalized against *GAPDH* mRNA levels and fold-change over non-stimulated shControl was calculated.

Discussion

In most cases the immune system succeeds in controlling hrHPV infections but this process takes time and requires the presence of strong IFNγ and TNFα-associated HPV-specific T cell responses [16]. Here we show that hrHPV-infected KCs resist the immune system by interfering with the regulation of intracellular growth and cell death programs of infected cells. Under normal circumstances these would be activated in response to the effector molecules of the adaptive immune system and function as a host defense mechanism to control viral spread [54]. Using a unique *in vitro* model we showed that hrHPV infection renders KCs resistant to IFNγ/TNFα-induced necroptosis and arrest of cell growth. HrHPV infection is associated with the upregulation of 9 methyltransferases, including EZH2, and downregulation of the expression of RIPK3 which results in an impaired induction of necroptosis by IFNγ/TNFα stimulation. Use of DZNep, a global inhibitor of methyltransferases and a pharmacological compound that depletes EZH2 [45, 46], restored the expression of RIPK3 and the sensitivity of hrHPV-infected KCs to IFNγ/TNFα-mediated necroptosis. Use of the catalytic EZH2 inhibitor GSK503 did restore RIPK3 expression, suggesting that either EZH2 is indirectly responsible for suppressing RIPK3 mediated necroptosis or that one or more of the other overexpressed methyltransferases are involved in the downregulation of RIPK3. Furthermore, hrHPV effectively downregulated the basal expression of the negative regulator of cell growth *IFITM1,* resulting in an impaired IFNγ-mediated increase in the expression of the anti-proliferative *RARRES1* gene and decrease of the proliferative gene *PCNA* as well as impaired arrest of cells in the S-phase. Knockdown of *IFITM1* with siRNA in normal KCs recapitulated the effects on *RARRES1* and *PCNA* expression and cell proliferation observed in hrHPV+KCs.

Apoptosis and necroptosis play an important role in controlling viral infections [54]. We did not observe any HPV-induced differences in the expression of caspase-8, FLIP or FADD, nor did we observe differences in cleavage of caspase-8 upon stimulation with IFNγ and TNFα. Notably, stimulation with IFNγ and TNFα did not lead to

activation of caspase-8, reflected by the absence of fully cleaved caspase-8 in undifferentiated normal or hrHPV-positive KCs. However, upon differentiation KCs become sensitive to Fas- and caspase-8-mediated apoptosis following stimulation with IFNγ and TNFα [29, 30]. In contrast, stimulation of undifferentiated KCs did result in necroptosis following an increase of RIPK3, most notably when both IFNγ and TNFα were used. The induction of necroptosis has shown to be important for the control of vaccinia virus [55] and herpes simplex virus type 1 [56]. Consequently, viruses have developed strategies to resist this immune control mechanism. The murine cytomegalovirus expresses the M45-encoded inhibitor of RIP activation (vIRA), that targets RIPK3 and disrupts RIPK1-RIPK3 interactions characteristic for necroptosis [57]. Rather than by interrupting necroptosis, hrHPV prevents the formation of the necrosome by reducing the levels of RIPK3 via the upregulation of histone methyltransferases.

The production of new HPV particles requires proliferation and differentiation of infected basal KCs. An arrest in cell proliferation, therefore, is an effective means to control viral infection. IFITM1 plays an essential role in the anti-proliferative action of IFNγ [47], thus lowering its expression – as observed in hrHPV-infected KCs – may allow viral escape. Indeed, hepatitis C virus was found to decrease the expression of *IFITM1* via the upregulation of mIR-130a in order to sustain its replication [58]. We showed that the basal expression of *IFITM1* is downregulated in hrHPV+KCs, but its downstream partner *RARRES1* is not. This might be explained by the fact that the basal expression of *RARRES1* in uninfected KCs is already low. The IFNγ-induced increase in expression of *IFITM1* and *RARRES1* requires signaling via the IFNGR1 and STAT1. Overexpression of EZH2 has been reported to suppress the expression of IFNGR1 in MYC- but not phosphatidylinositol 3-kinase (PI3K)-transformed cells, despite the fact that in both types of transformed cells EZH2 was overexpressed [59]. Notwithstanding the ectopic expression of EZH2 in hrHPV-positive KCs, there were no specific differences in the expression of IFNGR1 between non-infected and hrHPV-positive cells. This is in line with the observation that the PI3K pathway is a major target for the

hrHPV proteins [59, 60] and suggests that the overexpression of EZH2 does not play a role in the escape of hrHPV-positive cells at this level. It was previously reported that the HPV early proteins E6 and E7 downregulate the expression of STAT1 [4-6]. Our data confirm that infection with hrHPV decreases basal STAT1 protein levels in KCs but also show that hrHPV does not interfere with IFNγ-induced STAT1 activation *per se*, as reflected by STAT1 phosphorylation and increase in *RARRES1* and *IFITM1* expression. Still, as total STAT1 levels are lower in hrHPV+KCs, the reduced total amount of activated STAT1 may explain why in hrHPV+KCs the increase in *RARRES1* and *IFITM1* expression does not reach the levels observed in uninfected KCs. This is also demonstrated by the data showing that the effect of *IFITM1* knock-down on proliferation of uninfected KCs is not similar to what hrHPV has on KCs. Whilst the effect of IFITM1 in uninfected KCs is apparent and anti-proliferative, indicated by the retained expression of *PCNA* and *RARRES1* in KCs stimulated with a low dose of IFNγ when *IFITM1* was knocked-down, clearly the downregulation of STAT1 as well as the positive growth signals as delivered by hrHPV [52, 53] are missing in these cells. Hence, differences in IFNγ-stimulated arrest of proliferation are less noticeable. This shows that, whereas the decreased basal level of *IFITM1* is already providing resistance to the IFNγ-stimulated arrest of proliferation, the downregulation of STAT1 is likely to exaggerate this effect. Mechanistically, IFITM1 inhibits the phosphorylation of ERK and thereby regulates mitogen-activated protein (MAP) kinase signaling. Furthermore, IFITM1 mediates the de-phosphorylation of p53 at Thr55, resulting in increased p53 stability and transcriptional activity, and the upregulated expression of p21. Consequently, there is an arrest in cell cycle progression and hence a stop in proliferation [47]. This was also observed in this study and reflected by the retained *PCNA* expression when *IFITM1* was knocked-down in low dose IFNγ-stimulated KCs.

In conclusion, hrHPV controls proliferation by regulating the expression of (anti-)proliferative genes via STAT1 and IFITM1 and resists the induction of necroptotic cell death by downregulation of RIPK3 expression. This allows infected KCs to partly resist immune

pressure by IFNγ/TNFα and explains how hrHPV can partially evade the effector mechanisms of the immune system, which may ultimately lead to progression of hrHPV-induced lesions.

Conflict of Interest

The authors do not declare any conflict of interest.

Author Contributions

Design of study: JB and SVDB
Performed Experiments: WM, BT, RG
Delivered materials: EvE, CM
Analyzed data: WM, BT, RG, JB
Interpreted data: WM, BT, RG, EvE, CM, CJJM, JB, SvdB
Writing: WM, BT, CM, CJMM,JB, SvdB

Acknowledgments

WM is supported by a grant from the China Scholarship Council (201306240016). BT, JMB and SHvdB were supported by the Netherlands Organization for Health Research (NWO/ZonMw) TOP grant 91209012.

References

1. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nature reviews Cancer. 2002;2(5):342-50. doi: 10.1038/nrc798. PubMed PMID: 12044010.
2. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clinical science. 2006;110(5):525-41. doi: 10.1042/CS20050369. PubMed PMID: 16597322.
3. Tummers B, Burg SH. High-risk human papillomavirus targets crossroads in immune signaling. Viruses. 2015;7(5):2485-506. doi: 10.3390/v7052485. PubMed PMID: 26008697; PubMed Central PMCID: PMC4452916.
4. Chang YE, Laimins LA. Microarray analysis identifies interferon-inducible genes and Stat-1 as major transcriptional targets of human papillomavirus type 31. Journal of virology. 2000;74(9):4174-82. PubMed PMID: 10756030; PubMed Central PMCID: PMC111932.
5. Hong S, Mehta KP, Laimins LA. Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance. Journal of virology. 2011;85(18):9486-94. doi: 10.1128/JVI.05007-11. PubMed PMID: 21734056; PubMed Central PMCID: PMC3165741.
6. Nees M, Geoghegan JM, Hyman T, Frank S, Miller L, Woodworth CD. Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. Journal of virology. 2001;75(9):4283-96. doi: 10.1128/JVI.75.9.4283-4296.2001. PubMed PMID: 11287578; PubMed Central PMCID: PMC114174.
7. Zhou F, Chen J, Zhao KN. Human papillomavirus 16-encoded E7 protein inhibits IFN-gamma-mediated MHC class I antigen presentation and CTL-induced lysis by blocking IRF-1 expression in mouse keratinocytes. The Journal of general virology. 2013;94(Pt 11):2504-14. doi: 10.1099/vir.0.054486-0. PubMed PMID: 23956301.
8. Avvakumov N, Torchia J, Mymryk JS. Interaction of the HPV E7 proteins with the pCAF acetyltransferase. Oncogene. 2003;22(25):3833-41. doi: 10.1038/sj.onc.1206562. PubMed PMID: 12813456.
9. Bernat A, Avvakumov N, Mymryk JS, Banks L. Interaction between the HPV E7 oncoprotein and the transcriptional coactivator p300. Oncogene. 2003;22(39):7871-81. doi: 10.1038/sj.onc.1206896. PubMed PMID: 12970734.
10. Caberg JH, Hubert P, Herman L, Herfs M, Roncarati P, Boniver J, et al. Increased migration of Langerhans cells in response to HPV16 E6 and E7 oncogene silencing: role of CCL20. Cancer immunology, immunotherapy : CII. 2009;58(1):39-47. doi: 10.1007/s00262-008-0522-5. PubMed PMID: 18438663.
11. Havard L, Rahmouni S, Boniver J, Delvenne P. High levels of p105 (NFKB1) and p100 (NFKB2) proteins in HPV16-transformed keratinocytes: role of E6 and E7

oncoproteins. Virology. 2005;331(2):357-66. doi: 10.1016/j.virol.2004.10.030. PubMed PMID: 15629778.
12. Huang SM, McCance DJ. Down regulation of the interleukin-8 promoter by human papillomavirus type 16 E6 and E7 through effects on CREB binding protein/p300 and P/CAF. Journal of virology. 2002;76(17):8710-21. PubMed PMID: 12163591; PubMed Central PMCID: PMC136974.
13. Karim R, Tummers B, Meyers C, Biryukov JL, Alam S, Backendorf C, et al. Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response. PLoS pathogens. 2013;9(5):e1003384. doi: 10.1371/journal.ppat.1003384. PubMed PMID: 23717208; PubMed Central PMCID: PMC3662672.
14. Tummers B, Goedemans R, Jha V, Meyers C, Melief CJ, van der Burg SH, et al. CD40-Mediated Amplification of Local Immunity by Epithelial Cells Is Impaired by HPV. The Journal of investigative dermatology. 2014. doi: 10.1038/jid.2014.262. PubMed PMID: 24945092.
15. Tummers B, Goedemans R, Pelascini LP, Jordanova ES, van Esch EM, Meyers C, et al. The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NFkappaB activation. Nature communications. 2015;6:6537. doi: 10.1038/ncomms7537. PubMed PMID: 26055519; PubMed Central PMCID: PMC4382698.
16. van der Burg SH, Melief CJ. Therapeutic vaccination against human papilloma virus induced malignancies. Current opinion in immunology. 2011;23(2):252-7. doi: 10.1016/j.coi.2010.12.010. PubMed PMID: 21237632.
17. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine. 2009;361(19):1838-47. doi: 10.1056/NEJMoa0810097. PubMed PMID: 19890126.
18. van Poelgeest MI, Welters MJ, Vermeij R, Stynenbosch LF, Loof NM, Berends-van der Meer DM, et al. Vaccination against Oncoproteins of HPV16 for Noninvasive Vulvar/Vaginal Lesions: Lesion Clearance Is Related to the Strength of the T-Cell Response. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016;22(10):2342-50. doi: 10.1158/1078-0432.CCR-15-2594. PubMed PMID: 26813357.
19. Trimble CL, Morrow MP, Kraynyak KA, Shen X, Dallas M, Yan J, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. Lancet. 2015;386(10008):2078-88. doi: 10.1016/S0140-6736(15)00239-1. PubMed PMID: 26386540.
20. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, Lowik MJ, Berends-van der Meer DM, Essahsah F, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proceedings of the National Academy of Sciences of the United States of America.

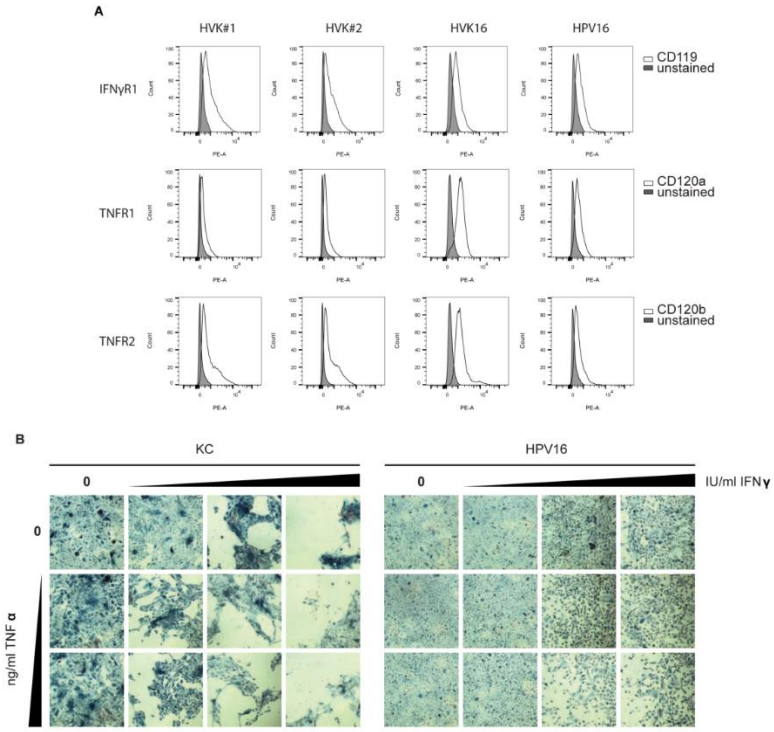
2010;107(26):11895-9. doi: 10.1073/pnas.1006500107. PubMed PMID: 20547850; PubMed Central PMCID: PMC2900675.
21. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nature reviews Immunology. 2005;5(5):375-86. doi: 10.1038/nri1604. PubMed PMID: 15864272.
22. Balachandran S, Adams GP. Interferon-gamma-induced necrosis: an antitumor biotherapeutic perspective. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2013;33(4):171-80. doi: 10.1089/jir.2012.0087. PubMed PMID: 23570383; PubMed Central PMCID: PMC3624769.
23. Thapa RJ, Nogusa S, Chen P, Maki JL, Lerro A, Andrake M, et al. Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(33):E3109-18. doi: 10.1073/pnas.1301218110. PubMed PMID: 23898178; PubMed Central PMCID: PMC3746924.
24. Cabal-Hierro L, Lazo PS. Signal transduction by tumor necrosis factor receptors. Cellular signalling. 2012;24(6):1297-305. doi: 10.1016/j.cellsig.2012.02.006. PubMed PMID: 22374304.
25. Detmar M, Orfanos CE. Tumor necrosis factor-alpha inhibits cell proliferation and induces class II antigens and cell adhesion molecules in cultured normal human keratinocytes in vitro. Archives of dermatological research. 1990;282(4):238-45. PubMed PMID: 2115318.
26. Hancock GE, Kaplan G, Cohn ZA. Keratinocyte growth regulation by the products of immune cells. The Journal of experimental medicine. 1988;168(4):1395-402. PubMed PMID: 2459297; PubMed Central PMCID: PMC2189076.
27. Saunders NA, Jetten AM. Control of growth regulatory and differentiation-specific genes in human epidermal keratinocytes by interferon gamma. Antagonism by retinoic acid and transforming growth factor beta 1. The Journal of biological chemistry. 1994;269(3):2016-22. PubMed PMID: 7904998.
28. Kono T, Tanii T, Furukawa M, Mizuno N, Taniguchi S, Ishii M, et al. Effects of human recombinant tumor necrosis factor-alpha (TNF-alpha) on the proliferative potential of human keratinocytes cultured in serum-free medium. The Journal of dermatology. 1990;17(7):409-13. PubMed PMID: 2229643.
29. Viard-Leveugle I, Gaide O, Jankovic D, Feldmeyer L, Kerl K, Pickard C, et al. TNF-alpha and IFN-gamma are potential inducers of Fas-mediated keratinocyte apoptosis through activation of inducible nitric oxide synthase in toxic epidermal necrolysis. The Journal of investigative dermatology. 2013;133(2):489-98. doi: 10.1038/jid.2012.330. PubMed PMID: 22992806.
30. Daehn IS, Varelias A, Rayner TE. T-lymphocyte-induced, Fas-mediated apoptosis is associated with early keratinocyte differentiation. Experimental dermatology. 2010;19(4):372-80. doi: 10.1111/j.1600-0625.2009.00917.x. PubMed PMID: 19645855.

31. Karim R, Meyers C, Backendorf C, Ludigs K, Offringa R, van Ommen GJ, et al. Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes. PloS one. 2011;6(3):e17848. doi: 10.1371/journal.pone.0017848. PubMed PMID: 21423754; PubMed Central PMCID: PMC3056770.
32. McLaughlin-Drubin ME, Christensen ND, Meyers C. Propagation, infection, and neutralization of authentic HPV16 virus. Virology. 2004;322(2):213-9. doi: 10.1016/j.virol.2004.02.011. PubMed PMID: 15110519.
33. Meyers C, Mayer TJ, Ozbun MA. Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. Journal of virology. 1997;71(10):7381-6. PubMed PMID: 9311816; PubMed Central PMCID: PMC192083.
34. Jones LJ, Gray M, Yue ST, Haugland RP, Singer VL. Sensitive determination of cell number using the CyQUANT cell proliferation assay. Journal of immunological methods. 2001;254(1-2):85-98. PubMed PMID: 11406155.
35. Mulay SR, Desai J, Kumar SV, Eberhard JN, Thomasova D, Romoli S, et al. Cytotoxicity of crystals involves RIPK3-MLKL-mediated necroptosis. Nature communications. 2016;7:10274. doi: 10.1038/ncomms10274. PubMed PMID: 26817517; PubMed Central PMCID: PMC4738349.
36. Ohnishi S, Okabe K, Obata H, Otani K, Ishikane S, Ogino H, et al. Involvement of tazarotene-induced gene 1 in proliferation and differentiation of human adipose tissue-derived mesenchymal stem cells. Cell proliferation. 2009;42(3):309-16. doi: 10.1111/j.1365-2184.2008.00592.x. PubMed PMID: 19250291.
37. Wu CC, Tsai FM, Shyu RY, Tsai YM, Wang CH, Jiang SY. G protein-coupled receptor kinase 5 mediates Tazarotene-induced gene 1-induced growth suppression of human colon cancer cells. BMC cancer. 2011;11:175. doi: 10.1186/1471-2407-11-175. PubMed PMID: 21575264; PubMed Central PMCID: PMC3112162.
38. Han J, Zhong CQ, Zhang DW. Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nature immunology. 2011;12(12):1143-9. doi: 10.1038/ni.2159. PubMed PMID: 22089220.
39. Wang L, Du F, Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. Cell. 2008;133(4):693-703. doi: 10.1016/j.cell.2008.03.036. PubMed PMID: 18485876.
40. He S, Wang L, Miao L, Wang T, Du F, Zhao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell. 2009;137(6):1100-11. doi: 10.1016/j.cell.2009.05.021. PubMed PMID: 19524512.
41. Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nature chemical biology. 2005;1(2):112-9. doi: 10.1038/nchembio711. PubMed PMID: 16408008.
42. Koo GB, Morgan MJ, Lee DG, Kim WJ, Yoon JH, Koo JS, et al. Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in

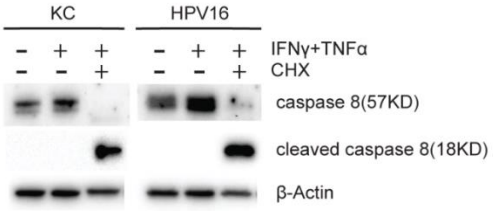
response to chemotherapeutics. Cell research. 2015;25(6):707-25. doi: 10.1038/cr.2015.56. PubMed PMID: 25952668; PubMed Central PMCID: PMC4456623.
43. Hyland PL, McDade SS, McCloskey R, Dickson GJ, Arthur K, McCance DJ, et al. Evidence for alteration of EZH2, BMI1, and KDM6A and epigenetic reprogramming in human papillomavirus type 16 E6/E7-expressing keratinocytes. Journal of virology. 2011;85(21):10999-1006. doi: 10.1128/JVI.00160-11. PubMed PMID: 21865393; PubMed Central PMCID: PMC3194988.
44. Sun S, Yu F, Zhang L, Zhou X. EZH2, an on-off valve in signal network of tumor cells. Cellular signalling. 2016;28(5):481-7. doi: 10.1016/j.cellsig.2016.02.004. PubMed PMID: 26876615.
45. Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes & development. 2007;21(9):1050-63. doi: 10.1101/gad.1524107. PubMed PMID: 17437993; PubMed Central PMCID: PMC1855231.
46. Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, et al. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. Molecular cancer therapeutics. 2009;8(6):1579-88. doi: 10.1158/1535-7163.MCT-09-0013. PubMed PMID: 19509260; PubMed Central PMCID: PMC3186068.
47. Yang G, Xu Y, Chen X, Hu G. IFITM1 plays an essential role in the antiproliferative action of interferon-gamma. Oncogene. 2007;26(4):594-603. doi: 10.1038/sj.onc.1209807. PubMed PMID: 16847454.
48. Ackrill AM, Reid LE, Gilbert CS, Gewert DR, Porter AC, Lewin AR, et al. Differential response of the human 6-16 and 9-27 genes to alpha and gamma interferons. Nucleic acids research. 1991;19(3):591-8. PubMed PMID: 1901407; PubMed Central PMCID: PMC333653.
49. Friedman RL, Manly SP, McMahon M, Kerr IM, Stark GR. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. Cell. 1984;38(3):745-55. PubMed PMID: 6548414.
50. Kelly JM, Gilbert CS, Stark GR, Kerr IM. Differential regulation of interferon-induced mRNAs and c-myc mRNA by alpha- and gamma-interferons. European journal of biochemistry / FEBS. 1985;153(2):367-71. PubMed PMID: 3935435.
51. Muller M, Laxton C, Briscoe J, Schindler C, Improta T, Darnell JE, Jr., et al. Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal transduction pathways. The EMBO journal. 1993;12(11):4221-8. PubMed PMID: 7693454; PubMed Central PMCID: PMC413716.
52. Fuentes-Gonzalez AM, Contreras-Paredes A, Manzo-Merino J, Lizano M. The modulation of apoptosis by oncogenic viruses. Virology journal. 2013;10:182. doi: 10.1186/1743-422X-10-182. PubMed PMID: 23741982; PubMed Central PMCID: PMC3691765.

53. Hamid NA, Brown C, Gaston K. The regulation of cell proliferation by the papillomavirus early proteins. Cellular and molecular life sciences : CMLS. 2009;66(10):1700-17. doi: 10.1007/s00018-009-8631-7. PubMed PMID: 19183849.
54. Mocarski ES, Upton JW, Kaiser WJ. Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways. Nature reviews Immunology. 2012;12(2):79-88. doi: 10.1038/nri3131. PubMed PMID: 22193709; PubMed Central PMCID: PMC4515451.
55. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell. 2009;137(6):1112-23. doi: 10.1016/j.cell.2009.05.037. PubMed PMID: 19524513; PubMed Central PMCID: PMC2727676.
56. Huang Z, Wu SQ, Liang Y, Zhou X, Chen W, Li L, et al. RIP1/RIP3 binding to HSV-1 ICP6 initiates necroptosis to restrict virus propagation in mice. Cell host & microbe. 2015;17(2):229-42. doi: 10.1016/j.chom.2015.01.002. PubMed PMID: 25674982.
57. Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. Cell host & microbe. 2010;7(4):302-13. doi: 10.1016/j.chom.2010.03.006. PubMed PMID: 20413098; PubMed Central PMCID: PMC4279434.
58. Bhanja Chowdhury J, Shrivastava S, Steele R, Di Bisceglie AM, Ray R, Ray RB. Hepatitis C virus infection modulates expression of interferon stimulatory gene IFITM1 by upregulating miR-130A. Journal of virology. 2012;86(18):10221-5. doi: 10.1128/JVI.00882-12. PubMed PMID: 22787204; PubMed Central PMCID: PMC3446586.
59. Wee ZN, Li Z, Lee PL, Lee ST, Lim YP, Yu Q. EZH2-mediated inactivation of IFN-gamma-JAK-STAT1 signaling is an effective therapeutic target in MYC-driven prostate cancer. Cell reports. 2014;8(1):204-16. doi: 10.1016/j.celrep.2014.05.045. PubMed PMID: 24953652.
60. Zhang L, Wu J, Ling MT, Zhao L, Zhao KN. The role of the PI3K/Akt/mTOR signalling pathway in human cancers induced by infection with human papillomaviruses. Molecular cancer. 2015;14:87. doi: 10.1186/s12943-015-0361-x. PubMed PMID: 26022660; PubMed Central PMCID: PMC4498560.

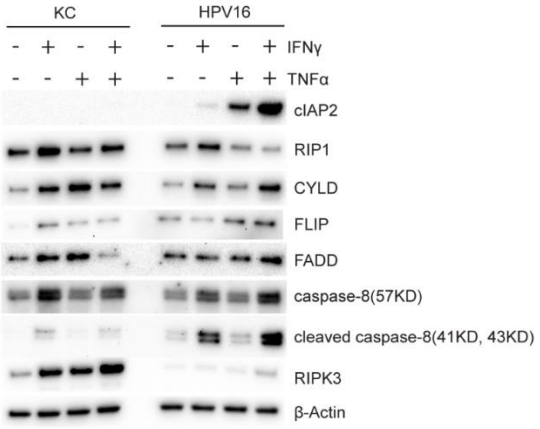
SUPPLEMENTARY INFORMATION



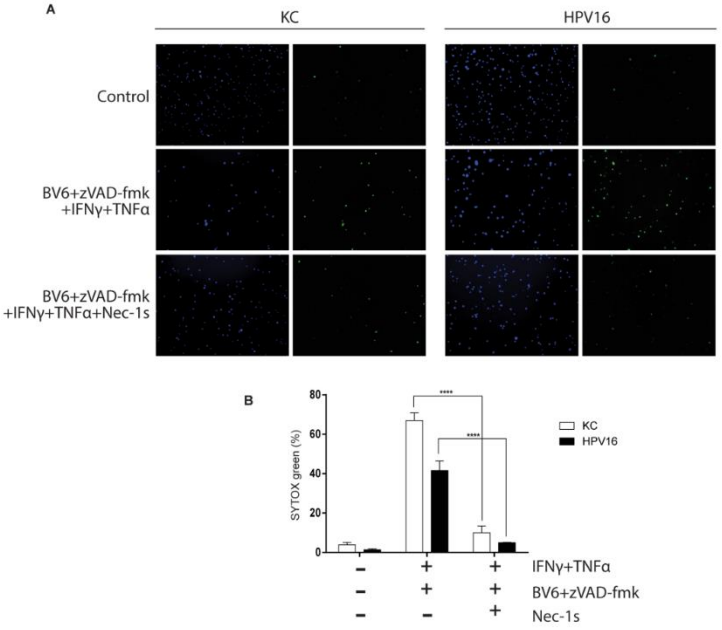
Supplemental Figure 1. The different effect of IFNγ and TNFα on HPV+ KCs is not due to alterations in cell surface expression of the receptors. (A) Cell surface expression of IFNγR1 (CD119), TNFR1 (CD120a) and TNFR2 (CD120b) of the indicated cell lines was analyzed by flow cytometry using phycoerythrin-conjugated antibodies. (B) Undifferentiated KCs (HFK) and HPV16+KCs (HPV16) were treated with increasing doses of IFNγ and/or TNFα for 72 hours after which cell confluency was monitored by MTT staining and phase-contrast microscopy as a measure for proliferation [60]. Representative microscopy pictures (4x magnification) are shown.



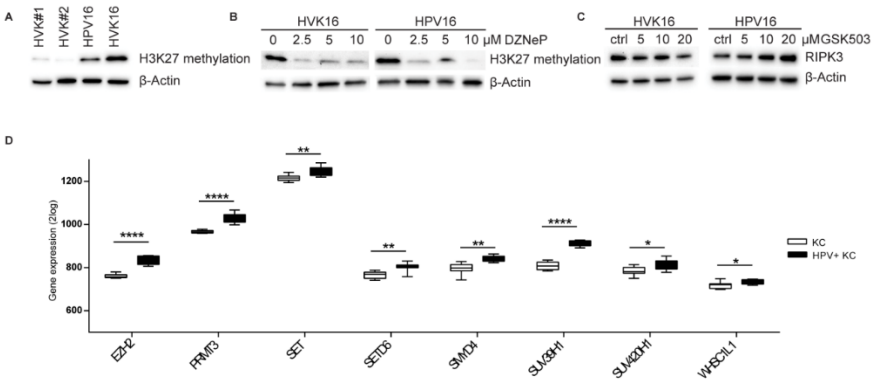
Supplemental Figure 2. Fully cleaved caspase-8 requires co-treatment of KCs with cycloheximide. Caspase-8 (57 kD) and fully cleaved caspase-8 (18 kD) protein level in KC and HPV16+KC stimulated with/without 50 IU/ml IFNγ, 30 ng/ml TNFα, and 100 μg/ml cycloheximide (CHX) for 48 hours, as measured by WB. ⊠-actin served as loading control.



Supplemental Figure 3. The expression of RIPK3 is enhanced by both IFNγ and TNFα.
The KC and HPV16+KCs were stimulated with 50 IU/ml IFNγ and/or 30 ng/ml TNFα for 24 hours. The protein expression levels of cIAP2, RIP1, CYLD, FLIP, FADD, caspase-8 (57 kD), cleaved caspase-8 (41 kD, 43 kD), and RIPK3 was measured by western blotting (WB) in whole cell extracts. β-actin served as loading control.



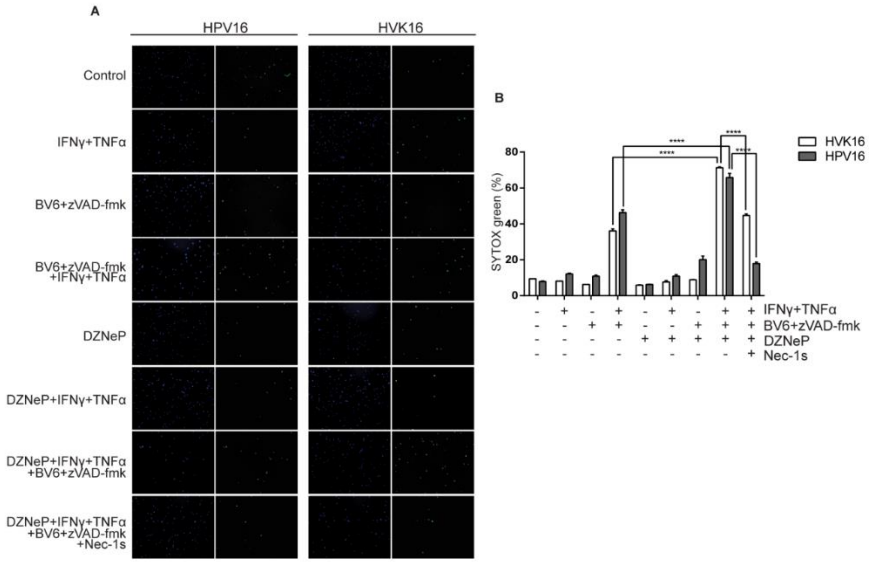
Supplemental Figure 4. The induction of IFNγ and TNFα-induced necroptosis is prevented by co-treatment with necrostatin-1s.
(A) Undifferentiated KCs (HVK#1)_and HPV16+KC (HPV16) were treated with or without 250 IU/ml IFNγ, 250 ng/ml TNFα, 5 μM BV6, 20 μM zVAD-fmk and/or 20 μM necrostatin-1s (Nec-1s) as indicated, for 48 hours. All cell nuclei were labelled with DAPI (blue fluorescence). Dead cells were stained using SYTOX green dead cell stain resulting in green fluorescent nuclei of dead cells.
(B) The number of dead cells among all cells was counted in multiple fields. The percentage of cell death was calculated. ****P < 0.001.



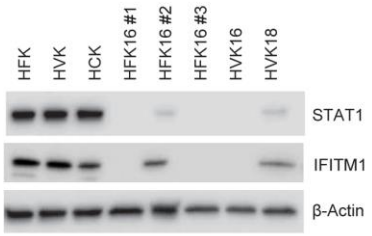
E

ASH1L	MLL5	SETD2	SMYD1	WHSC1L1
C5orf35	NSD1	SETD3	SMYD2	
DOT1L	NSD1	SETD4	SMYD3	
EHMT1	PRDM2	SETD5	SMYD4	
EHMT2	PRMT3	SETD6	SMYD5	
EZH1	PRDM9	SETD7	SUV39H1	
EZH2	SET	SETD8	SUV39H2	
MLL	SETBP1	SETDB1	SUV420H1	
MLL2	SETD1A	SETDB2	SUV420H2	
MLL3	SETD1B	SETMAR	WHSC1	

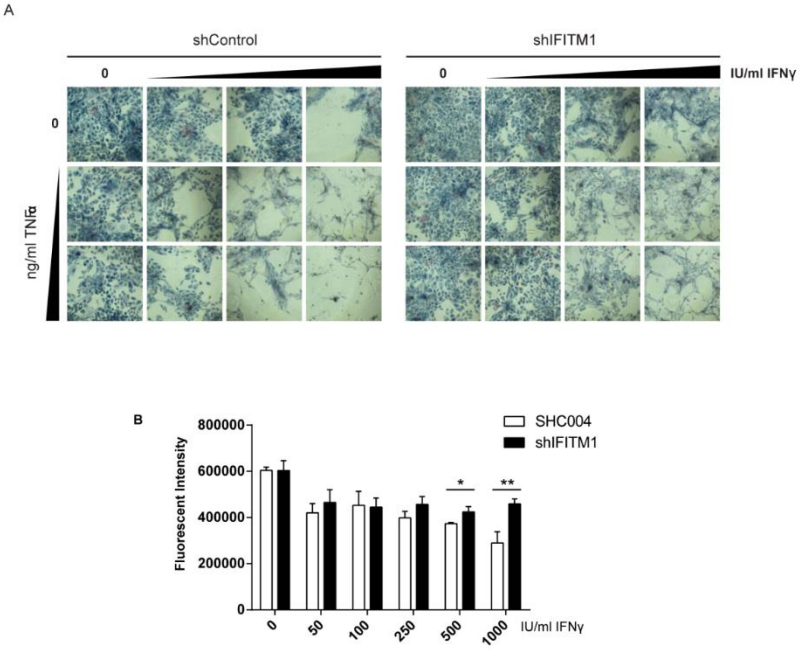
Supplemental Figure 5. Methylation of H3K27 in KCs and HPV16+ KCs and catalytic inhibition of EZH2 and expression of methyltransferases
(A) H3K27 methylation in KCs and HPV16+ KCs.
(B) The protein expression levels of H3K27 methylation were analysed in the two independent HPV16+KCs (HVK16, HPV16) either 72 hours after treatment with DZNeP.
(C) The protein expression levels of RIPK3 were analysed in the two independent HPV16+KCs (HVK16, HPV16) either 72 hours after treatment with GSK503, the catalytic inhibitor of EZH2, at indicated concentrations.
(D) Microarray gene expression values for methyltransferases significantly different expressed between 4 independent uninfected KCs and 4 independent hrHPV+KCs, represented in a box plot. * p<0.05.
(E) List of methyltransferases analyzed in microarray.



Supplemental Figure 6. Blocking global histone methylation rescues the induction of necroptosis by IFNγ and TNFα.
(A,B) The two HPV16+KCs (HVK16, HPV16) were treated with or without 10 μM DZNeP. After 24 hours, the cells were treated with 250 IU/ml IFN γ, 250 ng/ml TNFα, 5 μM BV6, 20 μM zVAD-fmk and/or 20 μM Nec-1s as indicated for 48 hours. All cell nuclei were labelled with DAPI (blue fluorescence). Dead cells were stained using SYTOX green dead cell stain resulting in green fluorescent nuclei of dead cells. (A) Representative example. (B) The percentage of cell death measured for each treatment was plotted for both HPV16+KC cultures. ****P < 0.001.



Supplemental Figure 7. Expression of STAT1 and IFITM1 in uninfected and hrHPV+ KCs.
STAT1 and IFITM1 protein levels in 3 independent KC, 4 independent HPV16+KC, and 1 HPV18+KC cultures as measured by western blotting (WB) in whole cell extracts. ⊠-actin served as loading control.



Supplemental Figure 8. Increased capacity of normal keratinocytes to resist IFNγ and TNFα-induced growth arrest after knock-down of IFITM1.
(A) Control and IFITM1 knock-down undifferentiated uninfected KCs were treated with IFNγ and/or TNFα for 96 hours after which cell confluency was monitored by MTT staining and phase-contrast microscopy as a measure for proliferation. Representative microscopy pictures (4x magnification) are shown.
(B) Control and IFITM1 knock-down undifferentiated uninfected KCs were treated with indicated doses of IFNγ for 96 hours after which the cellular DNA contents, as a measure of cell proliferation, was quantified using the CyQuant-NF assay. Knock-down of IFITM1 results in significant resistance to IFNγ mediated control of proliferation at the higher doses of IFNγ used.

Chapter 3

Control of immune escaped human papilloma virus is regained after therapeutic vaccination

Ma W, Melief CJ, van der Burg SH.

Current Opinion in Virology 2017, 23:16-22.

Control of immune escaped human papilloma virus is regained after therapeutic vaccination.

Wenbo Ma[a], Cornelis J.M. Melief[b], Sjoerd H. van der Burg[c,d]

[a] Department of Medical Oncology, Building 1, C7-141, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands. E-mail: W.Ma@lumc.nl
[b] ISA Pharmaceuticals, J.H. Oortweg 19, 2333 CH, Leiden, The Netherlands. E-mail: melief@isa-pharma.com
[c] Department of Medical Oncology, Building 1, C7-141, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands. E-mail: shvdburg@lumc.nl
[d] Corresponding author

Abstract

High-risk human papillomaviruses infect the basal cells of human epithelia. There it deploys several mechanisms to suppress pathogen receptor recognition signalling, impeding the immune system to control viral infection. Furthermore, infected cells become more resistant to type I and II interferon, tumour necrosis factor- and CD40 activation, via interference with downstream programs halting viral replication or regulating the proliferation and cell death. Consequently, some infected individuals fail to raise early protein-specific T-cell responses that are strong enough to protect against virus-induced premalignant disease and ultimately cancer. Therapeutic vaccines triggering a strong T-cell response against the early proteins can successfully be used to treat patients at the premalignant stage but combinations of different treatment modalities are required for cancer therapy.

Introduction

Progressive infections show split immunity to HPV late and early proteins

About 80% of sexually active individuals become infected with a high-risk HPV type (hrHPV). While most hrHPV infections (90%) are controlled within two years [1], viral persistence may lead to malignancies. The hrHPV are responsible for ~5% of all human cancers. Of the 14 different hrHPV types detected in cervical carcinoma, HPV16 and 18 are the most prevalent. HPV16 is the dominant type in all other HPV-induced cancers [2, 3].

HPV exclusively infect keratinocytes (KCs) in the basal layer of the epidermis and mucosal epithelia, through micro-wounds and abrasions. In the large majority of exposed but healthy individuals strong type 1 (IFNγ, TNFα, IL-2 producing) T-cell responses to the structural protein L1 as well as the early proteins E2, E6 and E7 are detected [4-7]. Stimulation of the L1-specific immune response most likely occurs via the uptake of virions, produced during the productive phase of the infection, by the Langerhans cells that reside in the epidermis. T-cell responses to L1 are detected in healthy individuals and in patients with premalignant lesions or cancer [7]. While they reflect a productive infection, they don't contribute to the control of viral infection as L1 is not expressed in the first few layers of the proliferating infected basal cells. In these layers, however, the early proteins E2, E6 and E7 are produced and immunity may be induced if these proteins are taken up by professional antigen presenting cells. However, type 1 T-cell responses to the early proteins are weak at best in patients with persistent infections.

The HPV infected skin expresses the cytosolic DNA sensors STING, AIM2 and IFI16. HPV DNA can trigger the latter two resulting in the secretion of IL-1β and IL-18 [8]. These cytokines mediate local and systemic immune responses to infection [9] and might be critical for early immune control of virus replication [10-12]. Hence, there is a period in which an HPV infection may trigger a protective T-cell response, dominated by CD4+ T-cells [5, 6, 12-14], but if this response is too weak or too late HPV may deploy several mechanisms to suppress the pathogen recognition receptor pathways [15-23] .

Importantly, as HPV infection does not cause viremia or cell lysis, either intact immune signalling or minor trauma to the lesion [24] is crucial to induce protective immunity.

Mechanisms used by HPV to prevent immune control

Basal KCs express several pattern-recognition receptors (PRR) that can recognize viral DNA or RNA (Figure 1). PRR ligation results in the production of type I interferon and pro-inflammatory cytokine production through signaling via interferon regulatory factor (IRF) and nuclear factor of kappa-light-chain-enhancer of activated B cells (NFκB) activating pathways. Several genome-wide transcription studies reported that hrHPV types have found means to suppress PRR- and type I IFN-induced signaling pathways [22]. Recently it was found that the cells in hrHPV-positive low-grade lesions display higher levels of E2 than normal hrHPV-infected cells, and this coincided with downregulation of STING [20]. Furthermore, hrHPV upregulated UCHL1, a deubiquitinase which was shown to inactivate TRAF3 and mediates the degradation of NEMO [15] and it may inhibit TLR9 expression [25]. Notably, prednisolone- and hydroxychloroquine-mediated downregulation of TLR7 and TLR9, respectively, is associated with HPV infections [26]. As a consequence, persistently hrHPV-infected cells will be less equipped to attract and activate the adaptive immune response via the production of interferons and cytokines (Figure 1). Especially, the secretion of the potent immune activating cytokine IL-1β is suppressed by hrHPV by targeting pro-IL-1β for destruction [27].

However, even when the immune system manages to mount a type 1 T-cell response it will be difficult for these T cells to control a persistent infection as hrHPV adapts the infected cells to become less sensitive to immune control mechanisms (Figure 1). The virus interferes with T-cell recognition via the reduction of MHC class I and II expression but also by affecting the downstream signalling pathways of CD40, and the TNFα and IFNγ receptors which normally will mitigate the infection by arresting cell proliferation and inducing cell death, but will also lead to amplification of the local immune

response via the direct (CD40, TNFα) and indirect (IFNγ) activation of NF B (Figure 1). Persistently hrHPV infected cells display lower levels of STAT1 but this does not completely impair signalling [28, 29]. Therefore, hrHPV also downregulate the interferon-induced transmembrane protein 1 (IFITM1) thereby preventing the upregulation of the antiproliferative gene RARRES1 [29] . A similar suppression of RARRES upregulation is noted after CD40 ligation [30]. In addition, hrHPV evades TNFα-induced cell death of infected cells by the downregulation of RIPK3, a crucial regulator of necroptosis[29]. Local amplification of immunity by the secretion of cytokines and the attraction of immune cells is dampened by hrHPV through an increased expression of interferon-related developmental regulator 1 (IFRD1), which attenuates the transcriptional activity of NFκB via deacetylation of RelA [31] as well as by interfering with downstream signalling of CD40, probably via the interaction of UCHL1 and TRAF6 [15, 30]. Finally, there is evidence that hrHPV-infected cells create a local immune suppressive microenvironment by altering the phenotype and function of local antigen dendritic cells [32] and the attraction of mast cells [33].

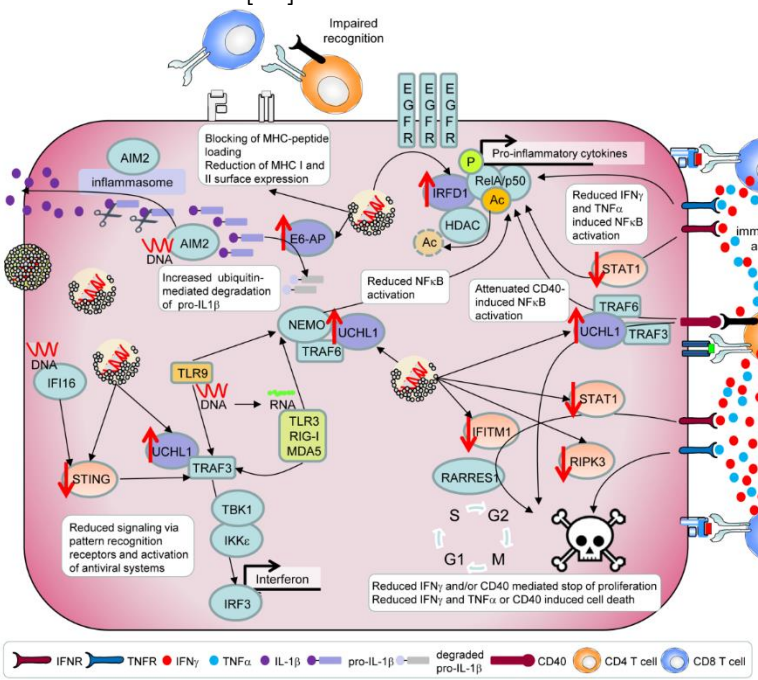


Figure 1 High-risk human papillomavirus deploys countermeasures to prevent immune control.

High-risk HPV can infect basal keratinocytes. The virus can be recognized by the pattern recognition receptors for viral DNA: IFI16, AIM2, TLR9 and for viral RNA: TLR-3, RIG-I, MDA-5. Most of these will activate interferon production via TRAF3-TBK1-IKK -IRF3 interactions but this is prevented by downregulation of STING and the upregulation of UCHL1, which inactivates TRAF3 via deubiquitination. UCHL1 also suppresses TLR9 and TLR3/RIG-I/MDA5-mediated activation of NF B via interaction with TRAF6 and degradation of NEMO. While viral DNA may activate the formation of the AIM2 inflammasome, required to cleave pro-IL1β into the potent immune activating cytokine IL-1β, the upregulation of E6-AP results in the ubiquitination of pro-IL1 targeting it for proteasomal degradation. Activated CD4+ type 1 T cells express CD40L and produce IFNγ and TNFα. Activation of CD40 and the IFNγ receptor (IFNR) result in proliferative arrest of cells, but this is impaired by the downregulation of STAT1 and IFITM1 (downstream of IFNR) and deactivation of TRAF3 (downstream of CD40) by UCHL1, with as result less upregulation of the antiproliferative gene RARRES1. RIPK3 is one of the key components in necroptosis, which is down-regulated by hrHPV, resulting in reduced IFNγ and TNFα induced necroptosis. High-risk HPV induces the overexpression of epidermal growth factor receptors (EGFR) and this increases the expression of IFRD1. IFRD1 mediates RelA K310 deacetylation thereby attenuating the transcriptional activity of NFκB. The resistance will be similar to CD8+ T-cell produced IFNγ and TNFα. Black arrows indicate the normal reactivity in the cell after stimulation. The purple proteins are upregulated and orange proteins are downregulated as a result of hrHPV infection.

A strong vaccine-induced type 1 T-cell response regains control of HPV-induced diseases

Therapeutic vaccines aim to stimulate strong type 1 helper T-cell and cytotoxic T-cell responses (Th1/CTL) to attack infected cells. They come in many flavours [34]and are also developed to treat HPV-induced diseases [35].
Clinical success has been obtained in women either with infected cells or with hrHPV-induced high-grade lesions. GTL001 in combination with the TLR7 agonist imiquimod topically applied to the vaccine site as adjuvant, stimulated E7-reactivity and a post-hoc analysis suggested increased and sustained clearance of HPV, albeit that the group size was small [36].
Four different types of vaccines were tested for their capacity to treat hrHPV-associated high-grade cervical lesions (CIN2-3). The DNA vaccine VGX-3100 was shown to induce strong E6/E7-specific Th1/CTL responses [37] and was subsequently tested in a large randomized placebo-controlled trial [38]. The spontaneous clearance rate of CIN2-3 was 30% and this was increased to 50% by vaccination. Post-hoc analyses revealed a relation between a clinical response and the strength of the vaccine-induced immune response [38]. Also the DNA vaccine GX-188E induced E6/E7-specific Th1/CTL responses that resulted in viral control and lesion regression in 7 out of 9 patients [39] while another (pnGVL4a-CRT/E7 DNA) failed to induce strong Th1/CTL reactivity or clinical reactivity exceeding the spontaneous clearance rate [40]. GLBL101c, an orally administered bacterial vector vaccine expressing HPV16 E7 protein [41] did not lead to overt systemic immunity but HPV-specific T-cells were detected in the cervix. A downgrade of disease stage was found in 5 of 13 patients [41] , just above the spontaneous clearance rate. Similarly, PepCan, an HPV16 E6 peptide-based vaccine with Candida skin test reagents as adjuvants induced T-cell reactivity in <50% of the subjects and there was no relation between immunity and lesion regression or an increase in clearance rate [42, 43]. The spontaneous clearance of HPV16-induced high-grade lesions of the vulva is less than 1.5% and treatment with the synthetic long peptide vaccine ISA101 considerably increased this percentage to more than 50% as shown in two subsequent medium-sized trials [44, 45]. Clinical reactivity was strongly related to the strength of the vaccine-induced Th1/CTL

response as found during the post-hoc analyses of the first trial [44, 46] and confirmed as pre-defined marker in the second trial [45].
The general observation from these trials is that if a strong Th1/CTL response is evoked one has the best chance for a clinical response. This fits with studies showing that hrHPV increases the resistance of infected cells to the effects of type 1 cytokine mediated signals but does not make them insensitive [15[31][31](31)(31)[31][31](Tummers, Goedemans et al. 2015)(Tummers, Goedemans et al. 2015)[31][31][31][31][31],23-25,[29, 31],42]. In addition, it should be appreciated that the viral gene expression changes during the progression of disease and this may impact on the immune evasive strategies deployed [22]. For example, STING expression is regained in progressive lesions, consistent with the loss of E2 protein expression [23] .
Currently 20 different ongoing trials focus on the treatment of premalignant or cancerous lesions (Table 1). Bearing in mind that local immune suppression hampers the efficacy of therapeutic vaccines [34] there are a couple of trials attracting the attention. Two trials try to circumvent general immune suppression by vaccinating patients during cancer surgery or after successful standard treatment, aiming to prevent recurrences (NCT00002916; NCT02405221). In three trials vaccination is combined with chemotherapeutics that may alleviate immune suppression mediated by regulatory T cells (NCT02865135) or myeloid cells (NCT 02526316; NCT02128126) [45, 47, 48]. Last but not least, activated T cells may express PD-1, which after engagement with PD-L1 on tumor cells or myeloid cells, suppresses their effector function. In one trial this is prevented by combining vaccination with the PD-1 blocking antibody nivolumab (NCT02426892).

Table 1 Current therapeutic vaccine trials

Vaccine	Goal	Disease stage	Status	NCT#
PDS0101	Safety, tolerability and pharmacodynam	Women with infection or CIN1	Recruiting	02065973

	ics of Versamune® + Peptides from HPV16 E6&E7			
VB10.16	Safety and immunogenicity of an HPV16 E6&E7 DNA vaccine targeted to antigen presenting cells	CIN2-3	Not recruiting	02529930
pnGVL4a-CRT/E7 DNA & topical imiquimod	Safety and efficacy of intralesional administration and Imiquimod treatment of lesion	CIN2-3	Recruiting	00988559
TA-HPV + Sig/E7/HSP70 DNA & topical imiquimod	Safety and efficacy of vaccination with Imiquimod treatment of lesion	CIN3	Recruiting	00788164
PepCan	Efficacy and safety of HPV16 E6 peptides & Candin adjuvant	CIN2-3	Recruiting	02481414
GX-188E	Determine recurrence of CIN and evaluation of long-term safety	CIN3	Recruiting	02411019
ISA101 & IFNα	Safety, immunogenicity	AIN2-3	Recruiting	01923116

as immune modulator	and efficacy of different intradermal doses HPV16 E6 and E7 synthetic long peptides with or without pegylated IFNα			
TA-HPV	Immunogenicity and impact on DFS when injected at time of surgery	Early cervical cancer	Completed	00002916
GM-CSF treated PBMC with E6/E7 peptides	Immunogenicity and efficacy of vaccination	Advanced or recurrent cancer	Completed	00019110
ADXS11	Immunogenicity and impact on 1 year survival of live-attenuated Listeria monocytogenes E6&E7 vaccine	Advanced or recurrent cancer	Suspended	01266460
BVAC-C	Safety and immunogenicity of recombinant HPV16/18 E6/E7 expressing Adenovirus-infected B-cells and monocytes	Advanced or recurrent cervical cancer	Recruiting	02866006
INO-3112	Safety and	Advanced or	Not	021729

	immunogenicity of VGX-3100 plus DNA-based immune activator encoded for IL-12	recurrent cancer	recruiting	11
INO-3112	Safety and immunogenicity when delivered by electroporation	Head and neck cancer	Not recruiting	02163057
ADXS11	Immunogenicity and toxicity of live-attenuated Listeria monocytogenes E6&E7 vaccine injected before surgery	Oropharyngeal cancer	Recruiting	02002182
ISA201	Biological activity of two HPV16 E6 specific peptides coupled to a Toll-like receptor ligand	Non-metastatic oropharyngeal cancer	Recruiting	02821494
P16_37-63 peptide in Montanide ISA51 & chemotherapy	Immunogenicity and safety of p16 peptide vaccination during cisplatin chemotherapy	HPV- and p16INK4a-positive cancer	Not recruiting	02526316
ISA101/101b	Safety and immunogenicity	Advanced or recurrent	Recruiting	02128126

in Montanide ISA51 & chemotherapy	of different doses HPV16 E6&E7 long peptides with or without pegylated IFNα as combination therapy with carboplatin and paclitaxel	HPV16-induced cancer		
DPX-E7 & chemotherapy	Safety and efficacy of a single HLA-A2-restricted HPV16 E7 epitope with metronomic cyclophosphamide	HPV-induced cancers	Not yet recruiting	02865135
ISA101 in Montanide ISA51 & Nivolumab	Phase 2 efficacy study of ISA101 with PD-1 checkpoint inhibition	HPV16-positive incurable cancers	Recruiting	02426892
TA-CIN & GPI-0100 adjuvant	Safety and feasibility of HPV16 L2-E6-E7 fusion protein with triterpene glycoside adjuvant	History of HPV16-positive cervical cancer	Not yet open	02405221

Conclusion

The high incidence of HPV infections, the quick clearance of infections in spite of HPV's stealthy behaviour, and the detection of early protein-specific T cells in most healthy subjects while seroconversion is low, indicates that in general pathogen recognition of hrHPV occurs after which a protective T-cell response is launched. The production of IL-1β may be crucial for the activation of a strong T-cell response during hrHPV infection. IL-1β is important for the acute phase response and it also enhances the expansion, differentiation and tissue localization of CD4+ and CD8+ T-cell responses [49, 50]. However, polymorphisms in the IL-1 gene [12, 51] and active downregulation of a network of IL-1β interconnected genes by hrHPV [16] as well as inhibition of IL-1β secretion at higher stages of disease [27] may stifle the development of protective type 1 T-cell responses in a minority of cases, with weak T-cell reactivity as result. In addition, hrHPV lowers the sensitivity of infected cells to key type 1 cytokines which otherwise will help infected cells to control the virus and it creates a local suppressive environment. This raises the bar for the type 1 T-cell responses to gain control of infection but therapeutic vaccines can stimulate type 1 HPV-specific T cell responses with a magnitude that readily exceeds the weak responses in patients and this is associated with regained control of hrHPV infection. In time, however, additional layers of immune suppression develop within the hrHPV-induced lesion necessitating combinations of vaccines with other treatment modalities to alleviate these suppressive mechanisms.

Acknowledgements

WM is supported by a grant from the China Scholarship Council (201306240016).

1. Veldhuijzen NJ, Snijders PJ, Reiss P, Meijer CJ, van de Wijgert JH. Factors affecting transmission of mucosal human papillomavirus. The Lancet Infectious diseases. 2010;10(12):862-74. doi: 10.1016/S1473-3099(10)70190-0. PubMed PMID: 21075056.

2. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. The New England journal of medicine. 2003;348(6):518-27. doi: 10.1056/NEJMoa021641. PubMed PMID: 12571259.

3. Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. International journal of cancer. 2007;121(3):621-32. doi: 10.1002/ijc.22527. PubMed PMID: 17405118.

4. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer research. 2003;63(3):636-41. PubMed PMID: 12566307.

5. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer research. 2002;62(2):472-9. PubMed PMID: 11809698.

6. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer research. 2004;64(15):5449-55. doi: 10.1158/0008-5472.CAN-04-0831. PubMed PMID: 15289354.

7. van Poelgeest MI, Nijhuis ER, Kwappenberg KM, Hamming IE, Wouter Drijfhout J, Fleuren GJ, et al. Distinct regulation and impact of type 1 T-cell immunity against HPV16 L1, E2 and E6 antigens during HPV16-induced cervical infection and neoplasia. International journal of cancer. 2006;118(3):675-83. doi: 10.1002/ijc.21394. PubMed PMID: 16108057.

8. Reinholz M, Kawakami Y, Salzer S, Kreuter A, Dombrowski Y, Koglin S, et al. HPV16 activates the AIM2 inflammasome in keratinocytes. Archives of dermatological research. 2013;305(8):723-32. doi: 10.1007/s00403-013-1375-0. PubMed PMID: 23764897.

9. Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JW, Joosten LA. IL-1beta processing in host defense: beyond the inflammasomes. PLoS pathogens. 2010;6(2):e1000661. doi: 10.1371/journal.ppat.1000661. PubMed PMID: 20195505; PubMed Central PMCID: PMC2829053.

10. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nature immunology. 2010;11(5):395-402. doi: 10.1038/ni.1864. PubMed PMID: 20351692; PubMed Central PMCID: PMC2887480.

11. Tavares MC, de Lima Junior SF, Coelho AV, Marques TR, de Araujo DH, Heraclio Sde A, et al. Tumor necrosis factor (TNF) alpha and interleukin (IL) 18 genes polymorphisms are correlated with susceptibility to HPV infection in patients with and without cervical intraepithelial lesion. Annals of human biology. 2016;43(3):261-8. doi: 10.3109/03014460.2014.1001436. PubMed PMID: 26079218.

12. Pontillo A, Bricher P, Leal VN, Lima S, Souza PR, Crovella S. Role of inflammasome genetics in susceptibility to HPV infection and cervical cancer development. Journal of medical virology. 2016;88(9):1646-51. doi: 10.1002/jmv.24514. PubMed PMID: 26945813.

13. Konopnicki D, Manigart Y, Gilles C, Barlow P, de Marchin J, Feoli F, et al. Sustained viral suppression and higher CD4+ T-cell count reduces the risk of persistent cervical high-risk human papillomavirus infection in HIV-positive women. The Journal of infectious diseases. 2013;207(11):1723-9. doi: 10.1093/infdis/jit090. PubMed PMID: 23463709.

14. Wang JW, Jiang R, Peng S, Chang YN, Hung CF, Roden RB. Immunologic Control of Mus musculus Papillomavirus Type 1. PLoS pathogens. 2015;11(10):e1005243. doi: 10.1371/journal.ppat.1005243. PubMed PMID: 26495972; PubMed Central PMCID: PMC4619818.

15. Karim R, Tummers B, Meyers C, Biryukov JL, Alam S, Backendorf C, et al. Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response. PLoS pathogens. 2013;9(5):e1003384. doi: 10.1371/journal.ppat.1003384. PubMed PMID: 23717208; PubMed Central PMCID: PMC3662672.

16. Karim R, Meyers C, Backendorf C, Ludigs K, Offringa R, van Ommen GJ, et al. Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes. PloS one. 2011;6(3):e17848. doi:

10.1371/journal.pone.0017848. PubMed PMID: 21423754; PubMed Central PMCID: PMC3056770.

17. Hasan UA, Bates E, Takeshita F, Biliato A, Accardi R, Bouvard V, et al. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. Journal of immunology. 2007;178(5):3186-97. PubMed PMID: 17312167.

18. Reiser J, Hurst J, Voges M, Krauss P, Munch P, Iftner T, et al. High-risk human papillomaviruses repress constitutive kappa interferon transcription via E6 to prevent pathogen recognition receptor and antiviral-gene expression. Journal of virology. 2011;85(21):11372-80. doi: 10.1128/JVI.05279-11. PubMed PMID: 21849431; PubMed Central PMCID: PMC3194958.

19. Hasan UA, Zannetti C, Parroche P, Goutagny N, Malfroy M, Roblot G, et al. The human papillomavirus type 16 E7 oncoprotein induces a transcriptional repressor complex on the Toll-like receptor 9 promoter. The Journal of experimental medicine. 2013;210(7):1369-87. doi: 10.1084/jem.20122394. PubMed PMID: 23752229; PubMed Central PMCID: PMC3698525.

20. Sunthamala N, Thierry F, Teissier S, Pientong C, Kongyingyoes B, Tangsiriwatthana T, et al. E2 proteins of high risk human papillomaviruses down-modulate STING and IFN-kappa transcription in keratinocytes. PloS one. 2014;9(3):e91473. doi: 10.1371/journal.pone.0091473. PubMed PMID: 24614210; PubMed Central PMCID: PMC3948877.

21. Lo Cigno I, De Andrea M, Borgogna C, Albertini S, Landini MM, Peretti A, et al. The Nuclear DNA Sensor IFI16 Acts as a Restriction Factor for Human Papillomavirus Replication through Epigenetic Modifications of the Viral Promoters. Journal of virology. 2015;89(15):7506-20. doi: 10.1128/JVI.00013-15. PubMed PMID: 25972554; PubMed Central PMCID: PMC4505635.

22. Tummers B, Burg SH. High-risk human papillomavirus targets crossroads in immune signaling. Viruses. 2015;7(5):2485-506. doi: 10.3390/v7052485. PubMed PMID: 26008697; PubMed Central PMCID: PMC4452916.

23. Poltorak A, Kurmyshkina O, Volkova T. Stimulator of interferon genes (STING): A "new chapter" in virus-associated cancer research. Lessons from wild-derived mouse models of innate immunity. Cytokine & growth factor reviews. 2016;29:83-91. doi: 10.1016/j.cytogfr.2016.02.009. PubMed PMID: 26980676.

24. Ekalaksananan T, Malat P, Pientong C, Kongyingyoes B, Chumworathayi B, Kleebkaow P. Local cervical immunity in women with low-grade squamous intraepithelial lesions and immune responses after abrasion. Asian Pacific journal of cancer prevention : APJCP. 2014;15(10):4197-201. PubMed PMID: 24935370.

25. Pacini L, Savini C, Ghittoni R, Saidj D, Lamartine J, Hasan UA, et al. Downregulation of Toll-Like Receptor 9 Expression by Beta Human Papillomavirus 38 and Implications for Cell Cycle Control. Journal of virology. 2015;89(22):11396-405. doi: 10.1128/JVI.02151-15. PubMed PMID: 26339055; PubMed Central PMCID: PMC4645680.

26. Yu SL, Chan PK, Wong CK, Szeto CC, Ho SC, So K, et al. Antagonist-mediated down-regulation of Toll-like receptors increases the prevalence of human papillomavirus infection in systemic lupus erythematosus. Arthritis research & therapy. 2012;14(2):R80. doi: 10.1186/ar3803. PubMed PMID: 22513098; PubMed Central PMCID: PMC3446454.

27. Niebler M, Qian X, Hofler D, Kogosov V, Kaewprag J, Kaufmann AM, et al. Post-translational control of IL-1beta via the human papillomavirus type 16 E6 oncoprotein: a novel mechanism of innate immune escape mediated by the E3-ubiquitin ligase E6-AP and p53. PLoS pathogens. 2013;9(8):e1003536. doi: 10.1371/journal.ppat.1003536. PubMed PMID: 23935506; PubMed Central PMCID: PMC3731255.

28. Hong S, Mehta KP, Laimins LA. Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance. Journal of virology. 2011;85(18):9486-94. doi: 10.1128/JVI.05007-11. PubMed PMID: 21734056; PubMed Central PMCID: PMC3165741.

29. Wenbo Ma BT, Edith M.G. van Esch, Renske Goedemans, Cornelis J.M. Melief, Craig Meyers, Judith M. Boer, Sjoerd H. van der Burg. Human Papillomavirus Downregulates the Expression of IFITM1 and RIPK3 to Escape from IFNγ- and TNFα-Mediated Antiproliferative Effects and Necroptosis. Frontiers in Immunology. 2016;7:496. doi: 10.3389/fimmu.2016.00496.

30. Tummers B, Goedemans R, Jha V, Meyers C, Melief CJ, van der Burg SH, et al. CD40-mediated amplification of local immunity by epithelial cells is impaired by HPV. The Journal of investigative dermatology. 2014;134(12):2918-27. doi: 10.1038/jid.2014.262. PubMed PMID: 24945092; PubMed Central PMCID: PMC4227541.

31. Tummers B, Goedemans R, Pelascini LP, Jordanova ES, van Esch EM, Meyers C, et al. The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NFkappaB activation. Nature communications. 2015;6:6537. doi: 10.1038/ncomms7537. PubMed PMID: 26055519; PubMed Central PMCID: PMC4382698.

32. Chandra J, Miao Y, Romoff N, Frazer IH. Epithelium Expressing the E7 Oncoprotein of HPV16 Attracts Immune-Modulatory Dendritic Cells to the Skin and Suppresses Their Antigen-Processing Capacity. PloS one. 2016;11(3):e0152886. doi: 10.1371/journal.pone.0152886. PubMed PMID: 27031095; PubMed Central PMCID: PMC4816461.

33. Bergot AS, Ford N, Leggatt GR, Wells JW, Frazer IH, Grimbaldeston MA. HPV16-E7 expression in squamous epithelium creates a local immune suppressive environment via CCL2- and CCL5- mediated recruitment of mast cells. PLoS pathogens. 2014;10(10):e1004466. doi: 10.1371/journal.ppat.1004466. PubMed PMID: 25340820; PubMed Central PMCID: PMC4207828.

34. van der Burg SH, Arens R, Ossendorp F, van Hall T, Melief CJ. Vaccines for established cancer: overcoming the challenges posed by immune evasion. Nature reviews Cancer. 2016;16(4):219-33. doi: 10.1038/nrc.2016.16. PubMed PMID: 26965076.

35. Vici P, Pizzuti L, Mariani L, Zampa G, Santini D, Di Lauro L, et al. Targeting immune response with therapeutic vaccines in premalignant lesions and cervical cancer: hope or reality from clinical studies. Expert review of vaccines. 2016;15(10):1327-36. doi: 10.1080/14760584.2016.1176533. PubMed PMID: 27063030.

36. Van Damme P, Bouillette-Marussig M, Hens A, De Coster I, Depuydt C, Goubier A, et al. GTL001, A Therapeutic Vaccine for Women Infected with Human Papillomavirus 16 or 18 and Normal Cervical Cytology: Results of a Phase I Clinical Trial. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016;22(13):3238-48. doi: 10.1158/1078-0432.CCR-16-0085. PubMed PMID: 27252412.

37. Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, Lee JC, et al. Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. Science translational medicine. 2012;4(155):155ra38. doi: 10.1126/scitranslmed.3004414. PubMed PMID: 23052295; PubMed Central PMCID: PMC4317299.

38. Trimble CL, Morrow MP, Kraynyak KA, Shen X, Dallas M, Yan J, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. Lancet. 2015;386(10008):2078-88. doi: 10.1016/S0140-6736(15)00239-1. PubMed PMID: 26386540.

39. Kim TJ, Jin HT, Hur SY, Yang HG, Seo YB, Hong SR, et al. Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. Nature communications. 2014;5:5317. doi: 10.1038/ncomms6317. PubMed PMID: 25354725; PubMed Central PMCID: PMC4220493.

40. Alvarez RD, Huh WK, Bae S, Lamb LS, Jr., Conner MG, Boyer J, et al. A pilot study of pNGVL4a-CRT/E7(detox) for the treatment of patients with HPV16+ cervical intraepithelial neoplasia 2/3 (CIN2/3). Gynecologic oncology. 2016;140(2):245-52. doi: 10.1016/j.ygyno.2015.11.026. PubMed PMID: 26616223; PubMed Central PMCID: PMC4724445.

41. Kawana K, Adachi K, Kojima S, Taguchi A, Tomio K, Yamashita A, et al. Oral vaccination against HPV E7 for treatment of cervical intraepithelial neoplasia grade 3 (CIN3) elicits E7-specific mucosal immunity in the cervix of CIN3 patients. Vaccine. 2014;32(47):6233-9. doi: 10.1016/j.vaccine.2014.09.020. PubMed PMID: 25258102.

42. Coleman HN, Greenfield WW, Stratton SL, Vaughn R, Kieber A, Moerman-Herzog AM, et al. Human papillomavirus type 16 viral load is decreased following a therapeutic vaccination. Cancer immunology, immunotherapy : CII. 2016;65(5):563-73. doi: 10.1007/s00262-016-1821-x. PubMed PMID: 26980480; PubMed Central PMCID: PMC4841729.

43. Greenfield WW, Stratton SL, Myrick RS, Vaughn R, Donnalley LM, Coleman HN, et al. A phase I dose-escalation clinical trial of a peptide-based human papillomavirus therapeutic vaccine with Candida skin test reagent as a novel vaccine adjuvant for treating women with biopsy-proven cervical intraepithelial neoplasia 2/3. Oncoimmunology. 2015;4(10):e1031439. doi: 10.1080/2162402X.2015.1031439. PubMed PMID: 26451301; PubMed Central PMCID: PMC4590015.

44. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine. 2009;361(19):1838-47. doi: 10.1056/NEJMoa0810097. PubMed PMID: 19890126.

45. van Poelgeest MI, Welters MJ, Vermeij R, Stynenbosch LF, Loof NM, Berends-van der Meer DM, et al. Vaccination against Oncoproteins of HPV16 for Noninvasive Vulvar/Vaginal Lesions: Lesion Clearance Is Related to the Strength of the T-Cell Response. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016;22(10):2342-50. doi: 10.1158/1078-0432.CCR-15-2594. PubMed PMID: 26813357.

46. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, Lowik MJ, Berends-van der Meer DM, Essahsah F, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(26):11895-9. doi: 10.1073/pnas.1006500107. PubMed PMID: 20547850; PubMed Central PMCID: PMC2900675.

47. Beyranvand Nejad E, van der Sluis TC, van Duikeren S, Yagita H, Janssen GM, van Veelen PA, et al. Tumor Eradication by Cisplatin Is Sustained by CD80/86-Mediated Costimulation of CD8+ T Cells. Cancer research. 2016;76(20):6017-29. doi: 10.1158/0008-5472.CAN-16-0881. PubMed PMID: 27569212.

48. van der Sluis TC, van Duikeren S, Huppelschoten S, Jordanova ES, Beyranvand Nejad E, Sloots A, et al. Vaccine-induced tumor necrosis factor-producing T cells synergize with cisplatin to promote tumor cell death. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(4):781-94. doi: 10.1158/1078-0432.CCR-14-2142. PubMed PMID: 25501579.

49. Ben-Sasson SZ, Hu-Li J, Quiel J, Cauchetaux S, Ratner M, Shapira I, et al. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(17):7119-24. doi: 10.1073/pnas.0902745106. PubMed PMID: 19359475; PubMed Central PMCID: PMC2678417.

50. Ben-Sasson SZ, Hogg A, Hu-Li J, Wingfield P, Chen X, Crank M, et al. IL-1 enhances expansion, effector function, tissue localization, and memory response of antigen-specific CD8 T cells. The Journal of experimental medicine. 2013;210(3):491-502. doi: 10.1084/jem.20122006. PubMed PMID: 23460726; PubMed Central PMCID: PMC3600912.

51. Dutta S, Chakraborty C, Mandal RK, Basu P, Biswas J, Roychoudhury S, et al. Persistent HPV16/18 infection in Indian women with the A-allele (rs6457617) of HLA-DQB1 and T-allele (rs16944) of IL-1beta -511 is associated with development of cervical carcinoma. Cancer immunology, immunotherapy : CII. 2015;64(7):843-51. doi: 10.1007/s00262-015-1693-5. PubMed PMID: 25893807.

Chapter 4

Intratumoral HPV16-specific T-cells Constitute a Type 1 Oriented Tumor Microenvironment to Improve Survival in HPV16-driven Oropharyngeal Cancer

Welters MJ, Ma W, Santegoets SJ, Goedemans R, Ehsan I, Jordanova KS, van Ham VJ, van Unen V, Koning F, van Egmond SI, van der Burg SH

Clin Cancer Res. 2018 Feb 1;24(3):634-647

Intratumoral HPV16-specific T-cells Constitute a Type 1 Oriented Tumor Microenvironment to Improve Survival in HPV16-driven Oropharyngeal Cancer

Marij J.P. Welters[1], Wenbo Ma[1], Saskia J.A.M. Santegoets[1], Renske Goedemans[1#], Ilina Ehsan[1], Ekaterina S. Jordanova[2†], Vanessa J. van Ham[1], Vincent van Unen[3], Frits Koning[3], Sylvia I. van Egmond[4], Pornpimol Charoentong[5‡], Zlatko Trajanoski[5], Lilly-Ann van der Velden[4$], and Sjoerd H. van der Burg[1*]

[1]Department of Medical Oncology, [2]Department of Pathology, [3]Department of Immunohematology and Blood Bank, and [4]Department of Otorhinolaryngology and Head and Neck Surgery, Leiden University Medical Centre, Leiden, the Netherlands. [5]Division for Bioinformatics, Innsbruck Medical University, Innsbruck, Austria. Additional author notes. Current address: [#]Genmab, Utrecht, the Netherlands. [†]Center for Gynaecologic Oncology, Free University Medical Center , Amsterdam, the Netherlands. [$]Department of Head and Neck Oncology and Surgery, Netherlands Cancer Institute-Antoni van Leeuwenhoek Hospital, Amsterdam. [‡]Department of Medical Oncology, National Center for Tumor Diseases, University Hospital Heidelberg, Heidelberg, Germany.

Running title: HPV-specific T-cells improve oropharyngeal cancer survival

Keywords: human papillomavirus, oropharyngeal cancer, clinical outcome, T-cell immunity, tumor microenvironment

Grant support: This study was financially supported by a grant from the Dutch Cancer Society 2014-6696 to SHvdB, LAvdV and MJPW.

Corresponding author: S.H. van der Burg, Department of Medical Oncology, Albinusdreef 2, 2233 ZA Leiden, the Netherlands. Phone: +31 71 5261180, Email: shvdburg@lumc.nl.

Conflict of interest: The authors declare no potential conflict of interests.

Author contributions: conception and design: M.J.P. Welters, L.A. van der Velden and S.H. van der Burg designed the study. S.I. van Egmond and L.A. van der Velden are the physicians treating the patients. M.J.P. Welters, W. Ma, R. Goedemans and I. Ehsan performed the immunological experiments. S.J.A.M. Santegoets, V.J. van Ham, V. van Unen and F. Koning were involved in the mass cytometry analysis. E. S. Jordanova conducted the immunofluorescent staining and analysis. P. Charoentong and Z. Trajanoski analyzed the TCGA database. M.J.P. Welters and S.H. van der Burg conducted the statistical analysis. M.J.P. Welters, W. Ma, S.J.A.M. Santegoets, L.A. van der Velden and S.H. van der Burg analyzed and interpreted the data. M.J.P. Welters, W. Ma, L.A. van der Velden and S.H. van der Burg wrote the manuscript. All authors approved the final manuscript.

Translational Relevance: A number of studies have reported that T-cells responding to the two oncoproteins E6 and E7 of high-risk human papillomavirus (HPV) can infiltrate the tumor microenvironment of patients with HPV-driven tumors and speculated that these T-cells might be important for tumor control. This is the first study that really addresses this question by measuring the T-cell response in the tumor, analysed the influence of these HPV16-specific T-cells on the microenvironment within the tumor and then waited for many years to define their impact on patient survival. Here we show how the presence of these HPV-specific T-cells is

associated with a completely different microenvironment and that intratumoral HPV-specific type 1 polarized T-cells provides HPV16-positive oropharyngeal cancer patients with a 37-fold higher chance to respond excellently to standard therapy, across all TNM stages. The results will fuel the discussion on de-intensification of the standard therapy and potential applicable forms of immunotherapy.

Abstract

Purpose: Human papilloma virus (HPV)-associated oropharyngeal squamous cell cancer (OPSCC) has a much better prognosis than HPV-negative OPSCC and this is linked to dense tumor immune infiltration. Since the viral antigens may trigger potent immunity, we studied the relationship between the presence of intratumoral HPV-specific T-cell responses, the immune contexture in tumor microenvironment and clinical outcome.

Experimental design: To this purpose an in-depth analysis of tumor-infiltrating immune cells in a prospective cohort of 97 HPV16-positive and -negative OPSCC patients was performed using functional T-cell assays, mass cytometry (CyTOF), flow cytometry and fluorescent immunostaining of tumor tissues. Key findings were validated in a cohort of 75 HPV16-positive OPSCC patients present in the publicly available cancer genomic atlas database.

Results: In 64% of the HPV16-positive tumors type 1 HPV16-specific T-cells were present. Their presence was not only strongly related to a better overall survival, a smaller tumor size and less lymph node metastases but also to a type I oriented tumor microenvironment, including high numbers of activated CD161+ T-cells, CD103+ tissue-resident T-cells, dendritic cells (DC) and DC-like macrophages.

Conclusions: The viral antigens trigger a tumor-specific T-cell response that shapes a favorable immune contexture for the response to standard therapy. Hence, reinforcement of HPV16-specific T-cell reactivity is expected to boost this process.

Introduction

The incidence of oropharyngeal squamous cell cancer (OPSCC) is rising, especially in younger adults [1]. Classically the development of OPSCC is related to p53 mutations, but currently more than half of all OPSCC are caused by a high-risk human papillomavirus, most often type 16 (HPV16) [1]. Although HPV-associated OPSCC are more often diagnosed with TNM stage III-IV, consisting of an earlier T stage and more advanced N stage, than HPV-negative OPSCC [2], they display a much better prognosis than HPV-negative tumors after (chemo)radiation therapy. This is independent of many common histopathological parameters [2, 3], but associated with the presence of a strong adaptive immune response gene signature [4] and dense tumor infiltration by activated CD4+ and CD8+ T-cells [3, 5, 6], suggesting a role for the adaptive immune system in the response to therapy. Notably, HPV-associated OPSCC express viral proteins and we have shown that they may function as tumor-specific antigens for OPSCC-infiltrating T-cells [7]. Clear evidence for a protective role of tumor-infiltrating HPV-specific T-cells in OPSCC, however, is lacking. Hence, it is important to evaluate if HPV-positive OPSCC are commonly infiltrated by HPV-specific T-cells, and specifically, how this pertains to the composition of the tumor microenvironment and survival. We purely focused on the analysis of HPV-specific T-cell reactivity within the tumor-infiltrating lymphocyte (TIL) population since detection of circulating HPV-specific T-cells might reflect a response to past infections [8], potentially even in other anatomical locations [8] and, thus, less relevant to our study. In case of such a relation, reinforcement of HPV-specific T-cell reactivity becomes highly attractive for treatment of OPSCC.

Materials and methods

Patients

Patients with histological confirmed OPSCC were included after signing informed consent. This study is part of a larger observational study P07-112 [7], approved by the local medical ethical committee

of the Leiden University Medical Center (LUMC) and in agreement with the Dutch law. Patient enrolment was from November 2007 until November 2015. Blood and tumor tissue samples were taken prior to treatment and handled as described previously [9] and in Supplementary Methods. Peripheral blood mononuclear cells (PBMCs) and tumor infiltrating lymphocytes (TILs) were stored until use. HPV typing and $p16^{ink4a}$ immunohistochemical staining was performed on former fixed paraffin embedded (FFPE) tumor sections at the department of pathology at the LUMC. Immunofluorescent staining of FFPE tumor sections for CD8 and Tbet was performed as described previously [10] and in Supplementary Methods. The patients received the standard-of-care treatment which could consist of surgery, radiotherapy, chemotherapy, treatment with monoclonal antibody or combinations hereof. Staging of the tumor was done according to the National Comprehensive Cancer Network (https://www.nccn.org/professionals). Patient characteristics are given in Supplementary Table S1.

Cancer cell lines.

The OPSCC cell lines were obtained from the University of Michigan (Ann Arbor, MI, USA) and called UM-SCC. We obtained UM-SCC4 (passage 22), UMC-SCC6 (passage 33), UM-SCC19 (passage 17) (all three HPV negative), and UM-SCC47 (passage 98) and UM-SCC104 (passage 15) (both HPV16-positive) in 2012. The cells were cultured in RPMI 1640 (Gibco/ Thermo Fisher Scientific (TFS) Bleiswijk, the Netherlands) with 10% Fetal Calf Serum (PAA laboratories; Pashing, Austria) and penicillin/streptomycin (TFS). Tumor cell supernatant (TSN) was prepared after 5 days of culture as described previously [11]. Microsatelite analysis was performed in July 2016 by BaseClear (Leiden, the Netherlands) to assure cell line authentication when the experiments were performed. Mycoplasma was tested on a monthly basis.

T-helper clones.

Clonal dilution was performed using the TILs from patient H68 as described previously [7]. Their HPV specificity and cytokine production was determined. This resulted in multiple CD4+ T-helper (Th) cell clones of which Th1 (clones 78 and 97), Th2 (clone 133) and Th17 (clones 12 and 103) were selected for the experiments. T-cell supernatant was obtained after stimulation with cognate HPV peptide loaded on with EBV immortalized B cells for 3 days.

TIL and tumor cell analyses

The phenotype and composition of dispersed tumors (and expanded TILs) was analyzed by flow [9, 12-15] and time of flight mass cytometry (CyTOF) [16] (Supplementary Methods). Supplementary Table S2 shows the 36 markers used for CyTOF analysis. The reactivity of TILs was determined in a 5-days proliferation assay [9] and by intracellular cytokine staining [15]. Supernatant from the proliferation test were subjected to cytokine analysis [15]. The effect of TSN on DC differentiation was determined phenotypically and functionally (cytokine/chemokine production) upon LPS or agonistic CD40 antibody stimulation in presence or absence of INF as described previously [11, 13] and in Supplementary Methods.

Treatment of tumor cells. Tumor cells were seeded (15000 – 27500 cells/well) in a flat bottom 96 well plate (Costar/TFS) and allowed to adhere overnight at 37^{o}C. The next day, the cells were incubated with the indicated concentrations of IFNγ and/or TNFα for 48 hours at 37^{o}C, followed by the MTT assay (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's protocol to determine the percentage of proliferating cells compared to the untreated cells (set at 100%) [13]. Tumor cells (70000 - 100000) were adhered in 24 well plate overnight as described above followed by treatment for 24 hours with a fixed dose of cisplatin (15 µg/mL) in the presence or absence of indicated concentrations TNFα (0-30 ng/mL). The cells were harvested and analysed for apoptosis by flow cytometry. In another experiment tumor cells prepared in 24 well plates were treated for 24 and 48 hours with IFNγ (250 IU/mL; Immunotools) and

TNFα (30 ng/mL) or 20% of supernatant obtained from Th1 (H68 clone 97), Th2 (H68 clone 133) or Th17 (H68 clone 103) cells with or without the addition of apoptosis inducer and cIAP1/2 interacting compound BV6 (5 µM smac mimetic; APExBIO, Houston, TX, USA) and pan-caspase inhibitor zVADfmk (20 µM FMK001, R&D systems, Minneapolis, MN, USA), together known to induce necroptosis [17-19]. Necrostatin (Nec)-1s (2263-1, Biovision, Milpitas, CA, USA) was added to the conditions used for UM-SCC19 to inhibit necroptosis via inhibition of RIP1K [14]. The treated tumor cells were harvested and subjected to SYTOX green staining to establish the percentage of dead cells and in parallel stained for flow-based apoptosis analysis using Annexin V (early apoptosis) and 7-AAD (late apoptosis). As indicated tumor cells were also analysed for RNA expression (quantative PCR) [14] and protein content (western blot) [14] (See also Supplementary Methods).

Statistical analysis

Unpaired parametric t test was used to determine the difference between various treatments of the cells from the UM-SCC tumor cell lines. Date of two groups of patients were analyzed using the unpaired non-parametric analysis (Man Whitney). Fisher Exact test was used to analyze categorical data in a contingency table. Data of the three groups of patients (p16-IR-; p16+IR-; p16+IR+) were analyzed using the unpaired non-parametric one-way ANOVA (Kruskal Wallis). Hazard ratio (HR) with a 95% confidence interval (CI95%) was calculated to determine the difference in survival curves. The non-parametric log-rank test (Mantel-Cox test) was done to compare the survival distribution of the two group of patients. In all cases a *P*-value of 0.05 and below was considered significant (*), P<0.01 (**) and P<0.001 (***) as highly significant.

Results

The majority of HPV16-positive OPSCC contain HPV16-specific Th1/Th17 cytokine producing T-cells

To interrogate the role of HPV-specific T-cells in OPSCC we prospectively assembled a cohort of 97 patients with OPSCC, 57 of which were HPV16 positive. Analysis of the patient characteristics showed the expected percentage of HPV-positive patients [2, 3] and the differences in smoking, N-stage and disease specific survival when compared to HPV-negative OPSCC (Fig. 1A, Supplementary Table S1), indicating that our patient cohort does not differ from those reported in literature.

From each patient both freshly obtained and FFPE tumor material was stored (Supplementary Fig. S1). The presence, proliferation and cytokine production of HPV16-specific and other OPSCC-infiltrating T-cells in the dissociated OPSCC were analyzed either directly or following a 2-4 weeks expansion period (Supplementary Fig. S1). Reactivity to the HPV16 E6 and/or E7 oncoproteins was detected directly *ex-vivo* in 6 out of 24 samples, and in 29 of 45 of the expanded TIL HPV16-positive cases. All directly *ex-vivo* detectable responses were confirmed in the expanded TIL. None of the 23 tested TIL cultures obtained from HPV-negative tumors displayed HPV-specific reactivity (Fig. 1B and 1C), showing the specificity of these type of TIL analyses [7] and demonstrating that HPV-specific T-cells only infiltrate HPV+ OPSCC.

Subsequently, supernatants taken from the HPV-reactive cultures were assessed for the presence of Th1 (IFNγ, TNFα, IL-2), Th2 (IL-4, IL-5, IL-10), and Th17 (IFNγ , IL-17) cytokines revealing a Th1/Th17 like profile (Fig. 1D). Flow cytometry analysis demonstrated that the population of activated and/or cytokine producing HPV-specific T-cells frequently comprised both CD4+ and CD8+ HPV-specific T-cells (Fig. 1E and Supplementary Fig. S2), which targeted multiple epitopes simultaneously (Fig. 1F), albeit that the percentage of HPV-specific cytokine producing CD4+ T-cells often was higher than that of CD8+ T-

cells (Fig. 1G). Thus, the majority of HPV16-positive OPSCC tumors are infiltrated by HPV16-specific CD4+ and CD8+ T-cells with a Th1/Th17 profile.

The mechanisms underlying the failure to detect an intratumoral HPV16-specific response can be manifold but a first requirement is the presence of sufficient quantities of antigen to stimulate T-cells. The expression of $p16^{INK4a}$ is a surrogate marker for overexpressed functionally active E7 oncoprotein [20]. Forty of the TIL tested HPV16-positive OPSCC tumors could be analyzed for $p16^{INK4a}$ overexpression and in contrast to immune responders (IR+), 7 out of the 15 immune non-responders (IR-) failed to show a positive staining (Supplementary Fig. S3A). Furthermore, tobacco smoking and in particular nicotine is known to impair the responsiveness of T-cells to antigenic stimulation [21]. While there were many patients with more than 10 pack years of smoking [2] (Supplementary Fig. S3B), this was not discriminative for the detection of HPV16-specific immunity (Supplementary Fig. S3C). Hence the failure to produce a T-cell reaction to HPV in HPV16-positive OPSCC most likely is due to the limited quantities of viral proteins available to the immune system.

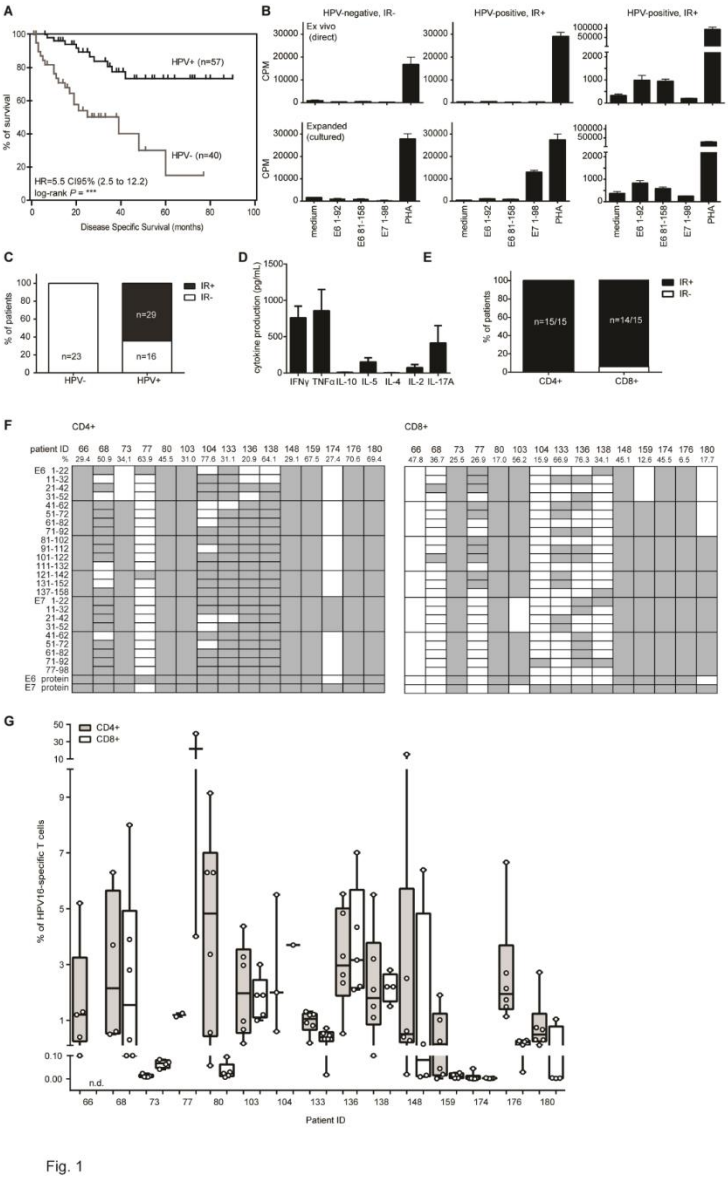


Fig. 1

Figure 1. HPV-driven oropharyngeal cancer induces HPV-specific T-cells and respond better to therapy. **A,** The Kaplan-Meier plot shows the survival of a cohort of 97 treated patients with oropharyngeal squamous cell carcinoma (OPSCC) divided by HPV status. **B,** Three

representative examples of freshly dispersed OPSCC as well as expanded (cultured) tumor infiltrating lymphocytes (TILs) for the same patient subjected to a proliferation assay (in triplicate wells) to determine the specificity of the TILs (shown as counts per minute (CPM) with standard error of mean (SEM)). Cells in medium only or stimulated with PHA served as a negative and positive control, respectively. **C,** In total 23 patients with a HPV-negative OPSCC and 45 patients with a HPV-positive OPSCC were tested in the proliferation assay as described in **B**. The percentage and number of patients showing an immune response (IR+) or not (IR-) is depicted. **D,** Cytokine production was determined in supernatants of HPV-reactive cultures in the proliferation assay. The average production of 21 cultured TILs is shown with SEM. **E,** The cultured TILs were stimulated with peptide pools or single peptides of the HPV16 E6 or E7 oncoprotein and analysed by multiparametric flow cytometry to determine the specific upregulation of activation markers (CD154 and CD137) and production of IFNγ, TNFα and IL-2 by CD4+ and CD8+ T-cells. The percentage and number of patients demonstrating an HPV-specific T-cell response are given. **F,** Heat map of the analysis as in **E** showing the specificity of HPV-specific responses (grey) to single peptides, pooled peptides and proteins of HPV16 E6 and E7 for each individual patient. The percentage of total CD4+ and CD8+ T-cells among TIL is indicated at the top of the heat map. **G,** The total frequency of HPV16-specific CD4+ and CD8+ T-cells in cultured TILs, indicated by the cumulative percentage of HPV-specific cytokine producing T-cells to each single peptide or pool, is shown for the individual patients. Box and whiskers are shown including the minimal and maximal value. N.d. is not detectable.

Tumor infiltration by HPV-specific T-cells correlates with high numbers of type 1 oriented T-cells and professional antigen presenting cells in the tumor

Based on the observation that the major component of OPSCC-infiltrating HPV-specific T-cells consists of CD4+ T-cells, and the known activity of tumor-specific CD4+ T-cells to recruit, activate and

sustain other immune cells [22, 23], we performed an in-depth analysis of the tumor microenvironment in the context of HPV-specific T-cell reactivity. Since the absence of overexpressed $p16^{INK4a}$ in HPV16-positive OPSCC may indicate that their development was not driven by the HPV oncoproteins [24], we separated the HPV16-positive patients into 3 groups: $p16^{INK4a}$-negative, IR-negative (p16-IR-); $p16^{INK4a}$-positive, IR-negative (p16+ IR-); and $p16^{INK4a}$-positive, IR-positive (p16+ IR+) patients.

An understanding of the general cytokine polarization in the tumors was obtained through analysis of cytokine production following the directly *ex-vivo* activation of all tumor-infiltrating T-cells using the mitogen phytohemagglutinin. Interestingly, the IFNγ/IL-17 cytokine polarization of HPV-specific T-cells was mirrored in the remainder of tumor-infiltrating cells (Supplementary Fig. S4). The production of IFNγ and IL-17 was lower in the p16+ IR- and the p16- IR- group. Moreover, the production of IL-5 was increased in the latter two groups suggesting a shift towards a more type 2 cytokine profile.

In addition, we quantified the number of type 1 polarized immune cells in the HPV16-positive tumors using immunohistochemistry for CD8 and the with IFNγ-production associated T-box transcription factor TBX21 (Tbet). The numbers of tumor-infiltrating Tbet+CD8+ T-cells and Tbet+CD8-negative T-cells, based on our flow cytometry data most likely CD4+ T-cells, correlated with an improved survival (Fig. 2A) and were particularly high when the OPSCC contained HPV-specific T-cells (Fig. 2B).

To comprehensively analyze the composition and phenotype of intratumoral immune cells directly *ex-vivo*, a validated panel of 36 antibodies adapted from a previous study [16] (Supplementary Table S2) was used in combination with mass cytometry (CyTOF) in 13 freshly dissociated OPSCC. This showed that the HPV16-positive OPSCC from HPV16 immune responder patients were stronger infiltrated with CD4+ and CD8+ T-cells (Fig. 2C and 2D) carrying an effector memory phenotype (Fig. 2E), whereas the HPV16-positive

OPSCC in which no HPV16-specific T-cell reactivity was detected, displayed a strong influx with B cells (Fig. 2D). NK cells, which may also infiltrate tumors and express Tbet, were virtually absent (Fig. 2D). In order to automatically discover stratifying biological signatures we used the CITRUS algorithm with a false discovery rate (FDR) of 1% resulting in 5 distinctive (groups of) populations of immune cells (Fig. 2F). It confirmed the differences in the percentages of tumor-infiltrating B cells and T-cells (Fig. 2G), but also revealed the presence of three subsets of T-cells that were present at significantly higher levels in HPV16 immune responders (Fig. 2H). Inspection of these subsets revealed two subsets of activated CD4+ T-cells and a subset of tissue-resident effector memory CD8+ T-cells expressing CD103 (Supplementary Fig. S5A and S5B). The two subsets of activated CD4+ T-cells expressed CD38, HLA-DR and PD1 but were separated on the basis of CD161 expression (Supplementary Fig. S5A and S5B). The CD161-negative subset of activated CD4+ T-cells had a high expression of CD25 but also expressed CD127, whereas the CD161+ subset displayed an intermediate expression of CD25, making it unlikely that these two populations reflected regulatory T-cells. Comparison of the tSNE plots of each patient clearly showed the almost exclusive presence of CD103+CD8+ T-cells in the IR-positive patient group (Supplementary Fig. S5C and S5D). Interestingly, part of the CD103+CD8+ T-cells also expressed CD161. There was no difference between the different patient groups with respect to the percentage of central memory CD161+CD4+ T-cells, but in each of the patients with an IR-positive HPV16-positive OPSCC a clearly visible effector memory CD161+CD4+ T-cell population was present (Supplementary Fig. S5C and S5D).

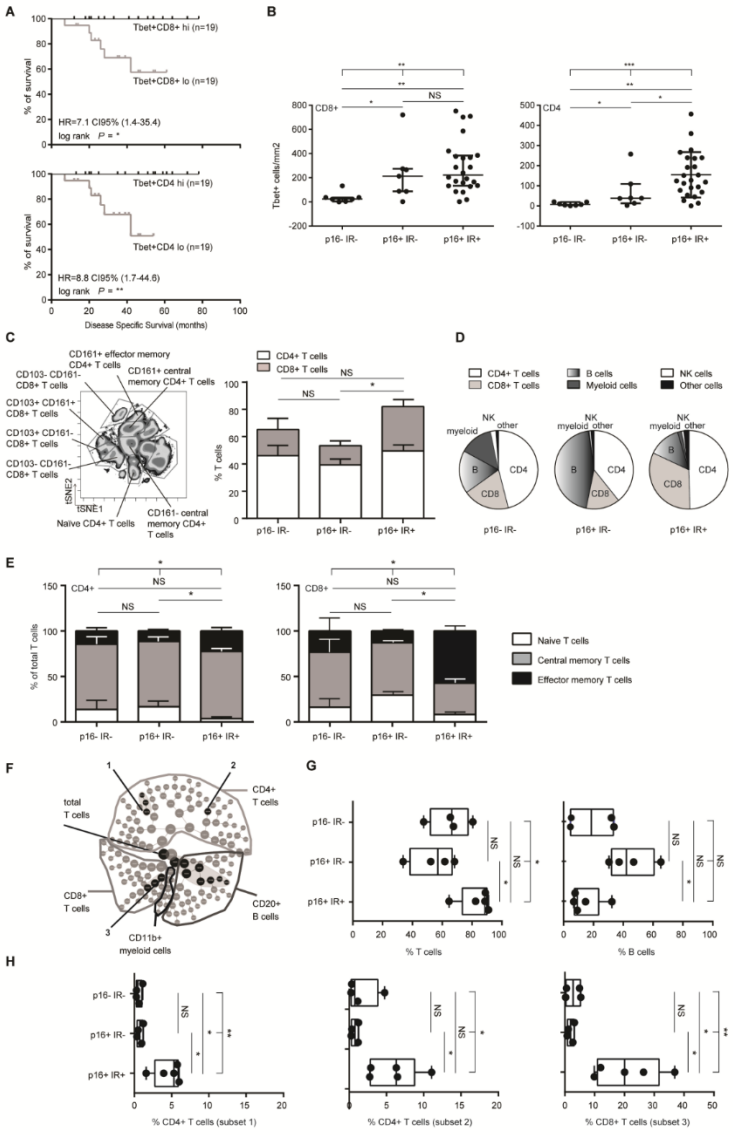


Fig. 2

Figure 2. HPV16-positive OPSCC harbouring HPV16-specific T-cells display a stronger and more activated immune infiltrate. A, The number of CD8+ and CD8- (CD4+) T-cells positive for Tbet per square

mm tumor as determined in OPSCC sections (5 high power fields per patient were counted). The 38 HPV16-positive OPSCC patients were grouped according to the number of Tbet-positive cells above (hi) or below (lo) the median counted number of these cells and plotted in a Kaplan-Meier for survival. **B,** The patients were grouped based on the $p16^{INK4a}$ expression of the tumor and the detection of an HPV-specific immune response (IR). The number of Tbet-positive T cells with each dot representing an individual patient sample and the median plus interquartile range is shown. Data of all three groups were analysed by Kruskal Wallis test. Data of two groups were analysed by unpaired non-parametric analysis (Man Whitney U test). **C,** The ViSNE plots visualize the high-dimensional CyTOF data in two dimensions. The different cell subsets are indicated. The frequency of CD4+ and CD8+ T-cells in the freshly dispersed OPSCC samples as determined by CyTOF are shown in the graph . Data are expressed as average frequencies (± SEM). The three groups differed significantly in their CD8+ T-cell frequency. **D,** Pie charts showing the composition of the immune cells and their relative contribution to the tumor microenvironment. **E,** The subdivision of the CD4+ and CD8+ frequencies (± SEM) into naïve, central memory and effector memory T-cells. Significant differences in the three groups for effector memory CD4+ and CD8+ T-cells and central memory CD8+ T-cells were found. **F,** CITRUS analysis visualized four main populations. The CD4+ T cell population included two subpopulations (indicated by the number 1 and 2) and the parental T-cell node is indicated as total T cells. **G,** The differences in frequency of T- and B-cells is depicted as box and whiskers (plus min-max) between the groups of patients. **H,** The frequency of the two subsets of CD4+ T-cells and the CD8+ T-cells (subset 3) as determined in **F** and similar to G. NS, not significant; **P*<0.05; ***P*<0.01 and ****P*<0.001.

In parallel, we analyzed the tumor microenvironment in a cohort of 75 HPV16-positive OPSCC patients present in the publicly available cancer genomic atlas (TCGA) database [25], using our previously published analytical strategy to estimate subpopulations of tumor-infiltrating immune cells [26]. Since CD4+ T-cells formed the major

component in IR-positive patients a gene set enrichment analysis (GSEA) of the TCGA RNA-sequencing data was performed to determine which immune cells were relatively enriched or depleted in HPV16-positive OPSCC with a high vs low CD4 gene expression (Fig. 3A). The results confirmed the enrichment of activated and effector memory T-cells, but also pointed at a potential enrichment in NK cells, activated DC and B cells as well as a decreased presence of MDSC in tumors with a high CD4 expression. Notably, an increased percentage of DCs/DC-like macrophages was observed among the HPV-responders when the dissociated HPV16-positive OPSCC of our cohort were analyzed by flow cytometry (n=18) or CyTOF (n=13) (Fig. 3B and 3C). *In vitro* experiments suggest that the increased percentages of these antigen presenting cells (APCs) is caused by the presence of the intratumoral IFNγ-producing HPV-specific T-cells. Analysis of the impact of two different HPV16-positive head and neck squamous cell carcinoma (HNSCC) cell lines [27, 28] on GM-CSF+IL-4 driven differentiation of monocytes to IL-12p70-producing DCs showed that tumor-secreted compounds skewed the monocytes towards type 2-like macrophages instead (Fig. 3D), that have a low capacity to produce IL-12p70 after CD40 ligation unless IFNγ was present (Fig. 3E). The resulting APCs now also produced the T-cell attracting chemokines CXCL9 and CXCL10 (Fig. 3F). Replacing IFNγ by the supernatant of genuine activated HPV-specific Th1 or Th17 T-cell clones (Supplementary Fig. S6A) also neutralized the M2-like macrophage skewing effect of the tumor cells (Supplementary Fig. S6B). A similar effect of HPV-specific Th1 and Th17 cytokines was observed on the direct M2-macrophage skewing effect of tumor cells (Supplementary Fig. S6C). In addition, the co-stimulatory molecules were upregulated.

Thus, the infiltration of OPSCC by HPV16-specific Th1/Th17 cells is associated with the presence of highly active tumor microenvironment consisting of a dense type 1 oriented immune cell infiltrate, known to favor immune-mediated control of cancer cells [29].

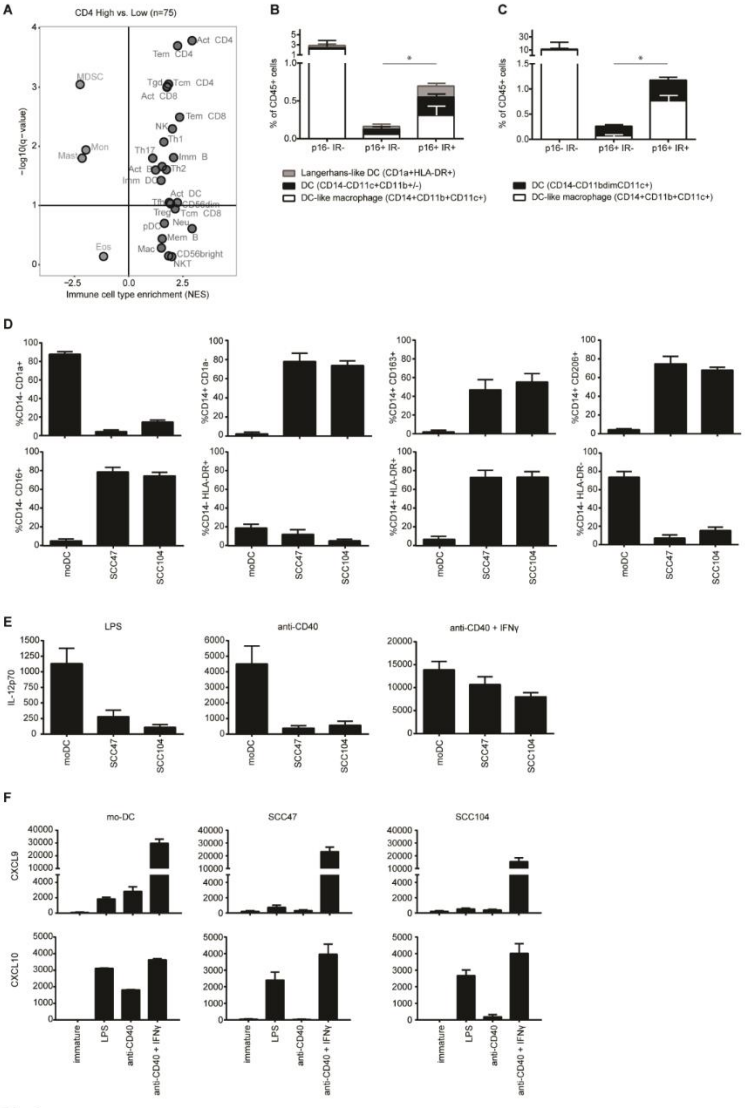


Fig. 3

Figure 3. HPV16-specific T-cell produced cytokines stimulate myeloid cells towards a type 1 phenotype. **A,** To identify immune cell types that are over-represented in HPV16-positive OPSCC with CD4+ T-cell

infiltrate a Gene Set Enrichment Analyses was performed on a cohort of 75 HPV16-positive OPSCC patients present in the publicly available TCGA database. The expression level of each gene was z-score normalized across all patients. For each patient (or group of patients) genes were then ranked in descending order according to their z-scores (mean of z-scores). The association was represented by a normalized enrichment score (NES). An immune cell type was considered enriched in a patient or group of patients when the false discovery rate (q-value) was ≤10%. The Volcano plot for the enrichment (blue) and depletion (beige) of immune cell types in CD4+ high vs CD4+ low HPV16-positive OPSCC is shown. **B,** The DCs, Langerhans-like DCs and DC-like macrophages in freshly dispersed OPSCC of 7 p16-IR-, 3 p16+IR- and 8 p16+IR+ patients were determined by flow cytometry (percentage of CD45+ cells ± SEM). **C,** As in **B** but analysed by mass cytometry (CyTOF; p16-IR- n=4; p16+IR- n=4; p16+IR+ n=5). Both in **B** and **C** significant differences in total DC population were observed between IR- and IR+ within the p16+ OPSCC patient group. **D,** Purified CD14+ cells from 5 healthy donors were cultured with IL-4 and GM-CSF for 6 days to differentiate them into monocytic DCs (moDC) in the presence/absence of 20% TSN obtained from UM-SCC47 or UM-SCC104 (both HPV16-positive OPSCC), stained and analysed by flow cytometry. The percentages (± SEM) of cells stained for the different marker combinations are shown. **E,** As in **D** for the 5 healthy donors after the cells have been stimulated for an additional 2 days with LPS, agonistic anti-CD40 antibody or the combination of this antibody with IFNγ. The IL-12p70 production (in pg/ml; mean ± SEM) is depicted. Non-stimulated cells (moDC) were taken along as negative control. **F,** As in **E** for the 5 healthy donors showing the production of CXCL9 and CXCL10 (in pg/ml; mean ± SEM) by these myeloid cells. NS, not significant; **P*<0.05; ***P*<0.01 and ****P*<0.001.

Type 1 cytokines influence tumor cell proliferation and synergize with cisplatin-induced cell death

The OPSCC-infiltrating HPV-specific CD4+ T-cells produced IFNγ and TNFα known to drive tumor cell senescence [30] and to synergize with platinum-based therapy to kill tumor cells [31]. We, therefore, studied if similar mechanisms could play a role in controlling oropharyngeal cancer cell growth by HPV-specific CD4 T-cells *in vitro*. We used our collection of 3 HPV-negative and 2 HPV16-positive HNSCC cell lines to analyze the expression of proteins involved in proliferation, apoptosis and necroptosis following stimulation with IFNγ and TNFα. All cell lines expressed the IFNGR and TNFR1 (and were responsive to IFNγ evidenced by the phosphorylation of STAT1, and to TNFα as shown by RelA phosphorylation (Supplementary Fig. S7A to S7C). Furthermore, they expressed the proteins required for apoptosis and necroptosis, although the HPV16-positive tumor cells lacked expression of the for necroptosis essential protein RIPK3 (Fig. 4A). Stimulation of the tumor cells with IFNγ and/or TNFα, or culture supernatant from antigen-stimulated HPV-specific Th1 or Th17 cells revealed a reduction in their proliferation (Fig. 4B and 4C) and an increase in the expression of the IFNγ responsive genes *IFITM1* and *RARRES*. Both genes are known to stop the proliferative process in cells [32, 33] (Fig. 4D and 4E), albeit that these effects differed per cell line tested. Expression analysis of the relation between IFNγ, *IFITM1* and *RARRES* in the TCGA cohort of HPV16-positive patients showed that especially IFNγ and *IFITM1* were co-expressed ($r = 0.475$; $P = 0.00060$), suggesting that IFNγ-induced arrest in proliferation occurs *in vivo.* In line with the RIPK3 expression only the HPV-negative cell lines were sensitive to necroptosis (Fig. 4F). Since cisplatin is the chemotherapeutic compound of choice for the treatment of OPSCC, the induction of cell death by increasing doses of TNFα in the presence of cisplatin was tested. The combination of TNFα and cisplatin resulted in an increased percentage of apoptotic tumor cells at 24 hours, specifically in the HPV-positive cell lines as in the HPV-negative cell lines no synergistic effect was observed (Fig. 4G) and a high percentage of death tumor cells at 48 hours (Supplementary Fig. S7D). These effects did not depend on necroptosis as inhibition with

necrostatin-1s did not prevent cell death (Supplementary Fig. S7D). Thus, apart from their role in changing the microenvironment, IFNγ and TNFα may also synergize with standard therapy in controlling tumor cell growth and form one of the underlying mechanisms explaining the good response rate of HPV-responding patients to chemoradiotherapy [2, 3].

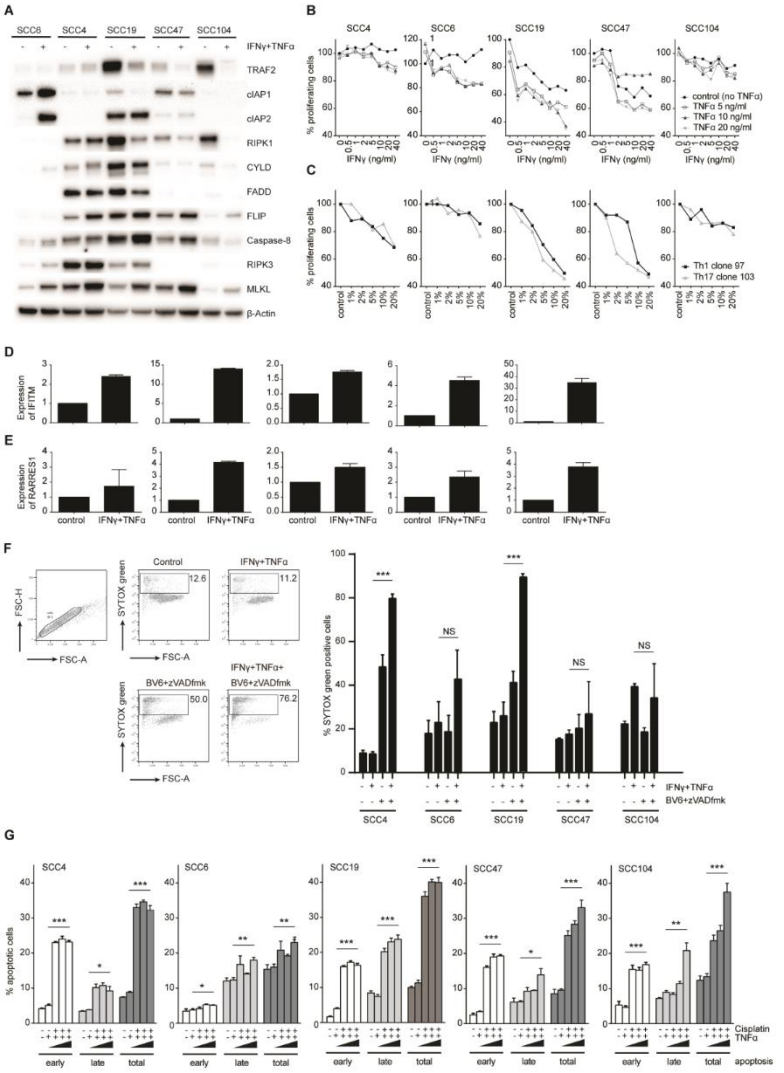


Fig. 4

Figure 4. Sensitivity and resistance of OPSCC cell lines to the anti-proliferative and cytotoxic effects of pro-inflammatory cytokines and/or chemotherapy. **A,** Protein expression of the indicated proteins

involved in the cell death pathway. **B,** Proliferation of tumor cells (from 5 different UM-SCC cell lines) treated with the indicated different concentrations of IFNγ and TNFα as determined by MTT assay with untreated cells were set at 100%. **C,** As in **B** but tumor cells were stimulated with different concentrations of culture supernatant from HPV-specific stimulated Th1 or Th17 clones. Tumor cells were left untreated (control) or treated with 50 IU/mL IFNγ and 30 ng/mL TNFα for 24 hours and the expression of **D,** *IFITM* and **E,** *RARRES1* was determined by RT-quantitative PCR and normalized to the GAPDH mRNA. The expression is given as mean (± SEM) for three independent experiments. **F,** The 5 different UM-SCC tumor cell lines were treated (in triplicate wells) for 48 hours with 250 IU/mL IFNγ and 30 ng/mL TNFα in the absence or presence of the necroptosis/apoptosis inducers BV6 (5 μM) and zVADfmk (20 μM). Untreated tumor cells were taken along as negative controls. Dead cells were stained positive using SYTOX green and the mean percentage (± SEM) are depicted. Unpaired T test analysis was performed between IFNγ+TNFα treatment with or without BV6+zVADfmk. **G,** The tumor cells were left untreated, treated for 24 hours with 30 ng/mL TNFα or with a fixed concentration of Cisplatin (15 mg/mL) plus increasing concentrations of TNFα (7.5, 15 or 30 ng/mL) as indicated by the triangle. The cells were stained for early apoptosis by Annexin-V and for late apoptosis by 7-AAD and analysed by flow cytometry. The mean percentage (± SEM) of the apoptotic cells in triplicate wells is shown. Total indicates the sum of percentage of both the early and late apoptotic cells. NS, not significant; **P*<0.05; ***P*<0.01 and ****P*<0.001.

Intratumoral activated effector memory CD161+CD4+ Th1/Th17 cells have a potential role in disease control

CD161+CD4+ T-cells are the dominant subtype of T-cells present in inflammatory diseases where CD4+ T-cells have an important role to drive acute inflammatory processes [34]. Hence, a similar role may be expected in the rejection of cancer cells. First, CD161 expression among freshly and *in vitro* expanded TILs was analyzed. A large

proportion of our fresh and *in vitro* expanded TILs expressed CD161. Importantly, *in vitro* expansion did not induce CD161 expression (Supplementary Fig. S8A). Subsequently, a flow cytometric analysis of 8 *in vitro* expanded TILs was performed to assess the HPV-specific component among these cells. On average the percentage of CD161+ CD4+ T-cells was 29% (Fig. 5A). The number of HPV-specific T-cells producing TNFα (Fig. 5B) was a bit higher than those producing IFNγ (Supplementary Fig. S8B) and on average 31% of the HPV-specific CD4+ T-cells expressed CD161 (Fig. 5B). This indicates that there was a sizeable CD161+ T-cell fraction among HPV-specific CD4+ T-cells in most of the patients and also that the distribution of CD161+ cells among these HPV-specific T-cells is similar to that of the total population.

Subsequently, we analyzed the survival of the 75 patients with HPV16-positive OPSCC in the publicly available TCGA database focusing on the expression of CD4, CD8, CD103 and CD161. A high expression of CD4, CD8 or CD161 was associated with better overall survival but this was not the case for CD103 expression (Fig. 5C-5F), albeit that the combined high expression of CD103 with CD8 resulted in a better segregation of the survival curves (Fig. 5G). This fits with the observation that the expression of CD8 and CD103 was not strongly correlated (r = 0,2559; *P* = 0 .0267) within this cohort. A high expression of CD161 with either high CD4 or CD8 expression was also associated with better survival (Fig. 5H and 5I). Notably, the populations of patients within the group seem to overlap completely. Indeed, these markers were highly co-expressed (CD4 and CD161: r = 0,8351; *P* = 0.00E00, and CD8 and CD161: r = 0.8363 ; *P* = 0.00E00), suggesting that they predominantly single out the same patients. Since the HPV-specific T-cells predominantly produced IFNγ, TNFα and IL-17 (Fig. 1D) we also analyzed the contribution of the respective gene expression levels to survival. Specifically, a high expression of IFNγ was associated with better survival while a similar trend was visible for IL-17 (Fig. 5J and 5K). Combinations of 2-3 cytokines did not result in better separation of the survival curves (Supplementary Fig. S8C-S8G).

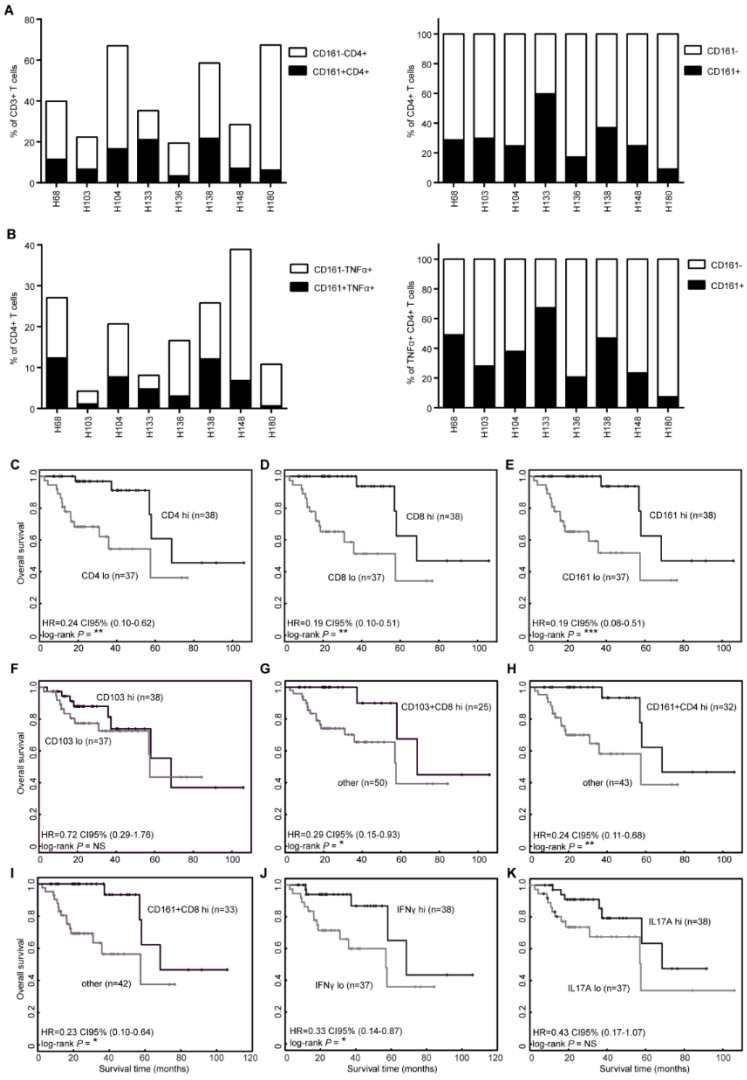


Fig. 5

Figure 5. Superior disease control correlates with the presence of intra-tumoral CD161+ T-cells. **A,** The proportion of CD161+CD4 T-cells among TIL (left) and the proportion of CD161+ cells among CD4+ T-

cells (right), where the frequencies of total CD4+ T-cells is set to 100%. **B,** As for **A** but now for the HPV-specific CD4+ T-cells producing TNFα upon stimulation with HPV16 E6 and/or E7 overlapping peptides. Kaplan-Meier survival plots of the 75 HPV16-positive OPSCC in the TCGA database grouped according to high and low gene expression using the median value of **C,** CD4, **D,** CD8, **E,** CD161 (KLRB1) and **F,** CD103. As in **C-F** but now patients are grouped according to a high expression of two indicated genes versus all others based on the median expression levels of **G,** CD103 and CD8, **H,** CD161 and CD4, **I,** CD161 and CD8. **J** and **K,** As in **C-F** for the expression of **J,** IFNγ and **K,** IL-17A. All graphs, the Hazard Ration (HR) with the 95% confidence interval (CI95%) as well as the log-rank test *P* value is given. NS, not significant; **P*<0.05; ***P*<0.01 and ****P*<0.001.

In combination with the above, these data suggest that a dense infiltration of HPV16-positive OSSC with IFNγ/IL-17 oriented CD4+ and/or CD8+ CD161+ T-cells, including the HPV16-specific T-cells, are important for superior disease control in HPV-driven OPSCC. Therefore, we analyzed the disease-specific survival of HPV-specific T-cell responders within the group of patients with HPV16-positive OPSCC. Patients with HPV-positive OPSCC displaying an HPV-specific T-cell reaction had a 37.8-fold (95%CI= 7.1 to 199.9) higher chance to respond to therapy when compared to patients with HPV16- positive OPSCC lacking such a T-cell reaction (Fig. 6A). Especially in stage III-IV HPV16- positive OPSCC, the local presence of an HPV16-specific T-cell response was a better prognostic parameter for a long survival after therapy than staging (Fig. 6B). The differences in survival between these two groups could not be attributed to a different cancer treatment (Supplementary Table 3). Intriguingly, also the T- and N-stage were on average lower in the immune responders (Fig. 6C and 6D), suggesting that HPV16-specific T-cells were especially present in patients with a better control of tumor growth.

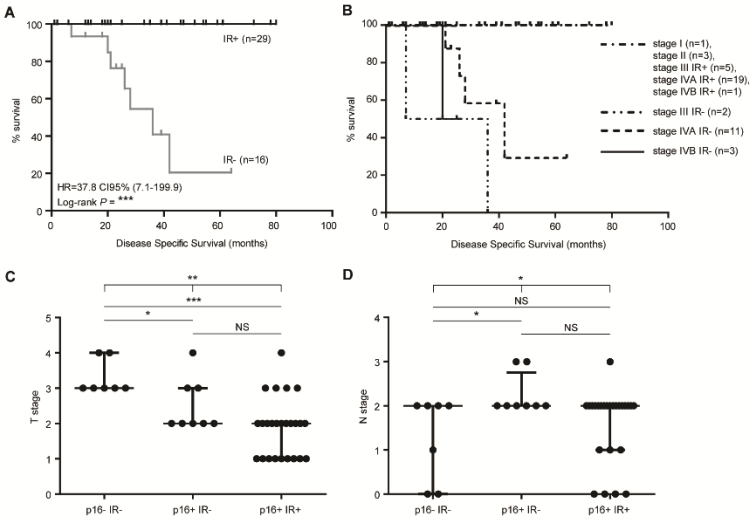


Fig. 6

Figure 6. HPV16-specific T-cells control tumor growth in HPV16-positive OPSCC. **A,** Kaplan-Meier survival curves showing the outcome of the 45 tested HPV16-positive OPSCC patients, who harboured HPV16-specific T-cells in their tumors (immune response positive, IR+) versus those who did not display an immune response (IR-). The HR with CI95% as well as log-rank *P* value is given. **B,** Kaplan-Meier survival curves when the 45 HPV16-positive OPSCC patients were plotted according to the stage of the disease. Staging was done according to the National Comprehensive Cancer Network. **C,** The group of 45 HPV16-positive OPSCC patients was split on the basis of $p16^{INK4a}$ expression in the tumor and having an immune response directed against HPV16 or not. The tumor size (T stage) is depicted for each individual patient in the three groups of patients. **D,** As in **C** but now for the involvement of lymph nodes (N stage). NS, not significant; **P*<0.05; ***P*<0.01 and ****P*<0.001.

Discussion

The improved clinical response of OPSCC patients to (chemo)radiotherapy has been associated with HPV and with a dense activated T-cell infiltrate but the role of the immune response against HPV in this still was not completely understood. Our findings demonstrate that the virally-derived E6 and E7 antigens make HPV-associated OPSCC highly visible to the immune system and unleashes an intratumoral HPV-specific T-cell response. These cells are poly-functional, detected among TIL in many of the patients, and have the CD161+ phenotype often found in acute rejection processes. They may locally facilitate the development of a clinically favorable tumor microenvironment because their presence is associated with a stronger influx of type 1 oriented CD4+ and CD8+ T-cells, as well as DCs and DC-like macrophages. Moreover, they produce cytokines which synergize with the platinum-based chemotherapy used to treat these patients and their detection is highly predictive for the response of patients to (chemo)radiotherapy.

HPV-specific CD4+ and CD8+ T-cells were detected in 64% of the TIL derived from HPV16- positive OPSCC, with a predominance of HPV-specific CD4+ T-cells, a result that closely matches an earlier study [35]. We show that these HPV-specific tumor-infiltrating T-cells as well as the other TIL predominantly produced IFNγ and IL-17, suggesting the presence of Th1 and Th17 cells. In view of the accepted roles of Th1/Th17 CD4+ and CD8+ T-cells in tumor control [36, 37], the detection of these cells in HPV16- positive OPSCC is likely to favor tumor control. Indeed, a high expression of IFNγ and to a lesser extend IL-17 in HPV16- positive OPSCC was associated with superior survival. Furthermore, the detection of HPV-specific T-cells singled out immunologically ‘’hot’’ tumors, with higher numbers of CD4+ and CD8+ T-cells expressing Tbet, effector memory T-cells, DCs and DC-like macrophages when compared to HPV16-positive OPSCC without HPV-specific T-cells. A dense tumor infiltration by T-cells [38] and DCs [39] as well as a predominant adaptive immune gene signature [4] have been associated with better survival in head and neck cancer, indicating that HPV-specific T-cell infiltrated tumors

possess the right type of inflammation. Last but not least, a dense infiltrate with T-cells is found more often in patients with superior local disease control [40] fitting with our observation that the group of patients with a tumor-specific immune response presented with a lower T- and N-stage.

Concomitant with the detection of HPV16-specific TIL, we found increased frequencies of CD161+ effector memory CD4+ and CD8+ TILs as well as CD8+CD103+ TILs. The intratumoral presence of CD8+CD103+ T-cells is a beneficial prognostic factor in a number of cancer types [41] and this would fit with the fact that we detected a high frequency of these cells specifically in T-cell inflamed tumors as well as with our analysis of the TCGA database, showing a survival advantage for HPV16-positive OPSCC patients with a strong expression of both CD8 and CD103. Earlier reports showed that CD161+ is predominantly detected on effector and central memory T-cells that produce IFNγ and/or TNFα [42], Th17 cells [43] and regulatory T-cells [44]. CD4+CD161+ T-cells can drive acute inflammatory processes [34], suggesting an important and similar role for them in cancer. Indeed, CD161 was among the top 10 of tumor leukocyte associated genes associated with positive prognosis for many human tumors [45]. In our study CD161 was expressed by tumor-specific IFNγ- and/or TNFα-producing CD4+ T-cells, higher frequencies of CD161 expressing CD4+ T-cells were detected in T-cell inflamed tumors and, finally, in the TCGA database the expression between CD161 and CD4 or CD8 was highly correlated and a high expression of these three genes was associated with a survival advantage for HPV16-positive OPSCC patients. Interestingly, mass cytometry showed that part of the CD8+CD103+ T-cells also expressed CD161.
In a large meta-analysis in head and neck cancer (MACH-NC) patients treated with radiotherapy alone have an overall 5-year survival of 27.2% whereas in patients receiving concomitant cisplatin chemotherapy and radiotherapy an improvement in overall survival of 6.5% is achieved [46]. Potentially this is explained by studies showing that platinum-based chemotherapy synergize with immune cell

produced IFNγ and TNFα in killing tumor cells [31], including OPSCC cells (this study). Due to the described cisplatin toxic side effects, dose reductions in cisplatin of 30% to 69% are often required for sustained concurrent chemo-radiotherapy treatment [47, 48] and de-intensification protocols for these patients are being discussed. This should not pose a major problem as lower doses of cisplatin still synergize with T-cell responses in animal tumor models [31].

Finally, the question surfaces whether reinforcement of HPV16-specific T-cell reactivity in patients with HPV16-positive OPSCC is warranted, not only to convert non-responders to HPV responders but also to boost existing responses. Clearly, the HPV16-positive OPSCC infiltrated by HPV16-specific T-cells meet the criteria of the cancer immunogram for immunotherapy [49]. The percentages of HPV-specific T-cells among TIL are respectable, however, not enough to mediate full tumor regression. In parallel to melanoma, where treatment with increased numbers of tumor-specific T-cells can mediate clinical responses, therapeutic vaccination is expected to increase the number of HPV16-specific T-cells and may result in clinical benefit for OPSCC patients. In view of the expression of PD-1, by the effector memory CD4+ and CD8+ T-cells (this study and [6]), and PD-L1 [50] in tumor tissue a combination of therapeutic vaccination and PD-1/PD-L1 blocking is expected to have the best outcome.

Acknowledgments: We thank the patients for participating in this study. Also we acknowledge C.E. van der Minne for her technical assistance.

References

1. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C. Epidemiology of Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma. J Clin Oncol. 2015;33(29):3235-42. Epub 2015/09/10. doi: 10.1200/jco.2015.61.6995. PubMed PMID: 26351338; PubMed Central PMCID: PMCPmc4979086.

2. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363(1):24-35. Epub 2010/06/10. doi: 10.1056/NEJMoa0912217. PubMed PMID: 20530316; PubMed Central PMCID: PMCPmc2943767.

3. Wansom D, Light E, Worden F, Prince M, Urba S, Chepeha DB, et al. Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. Arch Otolaryngol Head Neck Surg. 2010;136(12):1267-73. Epub 2010/12/22. doi: 10.1001/archoto.2010.211. PubMed PMID: 21173378; PubMed Central PMCID: PMCPmc3342998.

4. Thurlow JK, Pena Murillo CL, Hunter KD, Buffa FM, Patiar S, Betts G, et al. Spectral clustering of microarray data elucidates the roles of microenvironment remodeling and immune responses in survival of head and neck squamous cell carcinoma. J Clin Oncol. 2010;28(17):2881-8. Epub 2010/05/12. doi: 10.1200/jco.2009.24.8724. PubMed PMID: 20458058.

5. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nel H, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clin Cancer Res. 2006;12(2):465-72. Epub 2006/01/24. doi: 10.1158/1078-0432.ccr-05-1886. PubMed PMID: 16428488.

6. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, et al. PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. Cancer Res. 2013;73(1):128-38. Epub 2012/11/09. doi: 10.1158/0008-5472.can-12-2606. PubMed PMID: 23135914.

7. Heusinkveld M, Goedemans R, Briet RJ, Gelderblom H, Nortier JW, Gorter A, et al. Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer. Int J Cancer. 2012;131(2):E74-85. Epub 2011/10/25. doi: 10.1002/ijc.26497. PubMed PMID: 22020783.

8. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral

encounter. Cancer Res. 2003;63(3):636-41. Epub 2003/02/05. PubMed PMID: 12566307.

9. Welters MJ, van der Sluis TC, van Meir H, Loof NM, van Ham VJ, van Duikeren S, et al. Vaccination during myeloid cell depletion by cancer chemotherapy fosters robust T cell responses. Science translational medicine. 2016;8(334):334ra52. Epub 2016/04/15. doi: 10.1126/scitranslmed.aad8307. PubMed PMID: 27075626.

10. Punt S, Dronkers EA, Welters MJ, Goedemans R, Koljenovic S, Bloemena E, et al. A beneficial tumor microenvironment in oropharyngeal squamous cell carcinoma is characterized by a high T cell and low IL-17(+) cell frequency. Cancer Immunol Immunother. 2016;65(4):393-403. Epub 2016/02/24. doi: 10.1007/s00262-016-1805-x. PubMed PMID: 26899388; PubMed Central PMCID: PMCPmc4826411.

11. Heusinkveld M, de Vos van Steenwijk PJ, Goedemans R, Ramwadhdoebe TH, Gorter A, Welters MJ, et al. M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. J Immunol. 2011;187(3):1157-65. Epub 2011/06/29. doi: 10.4049/jimmunol.1100889. PubMed PMID: 21709158.

12. Dijkgraaf EM, Santegoets SJ, Reyners AK, Goedemans R, Wouters MC, Kenter GG, et al. A phase I trial combining carboplatin/doxorubicin with tocilizumab, an anti-IL-6R monoclonal antibody, and interferon-alpha2b in patients with recurrent epithelial ovarian cancer. Ann Oncol. 2015;26(10):2141-9. Epub 2015/07/29. doi: 10.1093/annonc/mdv309. PubMed PMID: 26216383.

13. Dijkgraaf EM, Heusinkveld M, Tummers B, Vogelpoel LT, Goedemans R, Jha V, et al. Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment. Cancer Res. 2013;73(8):2480-92. Epub 2013/02/26. doi: 10.1158/0008-5472.can-12-3542. PubMed PMID: 23436796.

14. Ma W, Tummers B, van Esch EM, Goedemans R, Melief CJ, Meyers C, et al. Human Papillomavirus Downregulates the Expression of IFITM1 and RIPK3 to Escape from IFNgamma- and TNFalpha-Mediated Antiproliferative Effects and Necroptosis. Front Immunol. 2016;7:496. Epub 2016/12/07. doi: 10.3389/fimmu.2016.00496. PubMed PMID: 27920775; PubMed Central PMCID: PMCPmc5118436.

15. van Poelgeest MI, Visconti VV, Aghai Z, van Ham VJ, Heusinkveld M, Zandvliet ML, et al. Potential use of lymph node-derived HPV-specific T cells for adoptive cell therapy of cervical cancer. Cancer Immunol Immunother. 2016;65(12):1451-63. Epub 2016/09/14. doi: 10.1007/s00262-016-1892-8. PubMed PMID: 27619514; PubMed Central PMCID: PMCPmc5099359.

16. van Unen V, Li N, Molendijk I, Temurhan M, Hollt T, van der Meulen-de Jong AE, et al. Mass Cytometry of the Human Mucosal Immune System Identifies Tissue- and Disease-Associated Immune Subsets. Immunity. 2016;44(5):1227-39. Epub 2016/05/15. doi: 10.1016/j.immuni.2016.04.014. PubMed PMID: 27178470.

17. Cristofanon S, Abhari BA, Krueger M, Tchoghandjian A, Momma S, Calaminus C, et al. Identification of RIP1 as a critical mediator of Smac mimetic-mediated sensitization of glioblastoma cells for Drozitumab-induced apoptosis. Cell Death Dis. 2015;6:e1724. Epub 2015/04/17. doi: 10.1038/cddis.2014.592. PubMed PMID: 25880091; PubMed Central PMCID: PMCPmc4650534.

18. Siegmund D, Kums J, Ehrenschwender M, Wajant H. Activation of TNFR2 sensitizes macrophages for TNFR1-mediated necroptosis. Cell Death Dis. 2016;7(9):e2375. Epub 2016/12/03. doi: 10.1038/cddis.2016.285. PubMed PMID: 27899821; PubMed Central PMCID: PMCPmc5059883.

19. Opel D, Schnaiter A, Dodier D, Jovanovic M, Gerhardinger A, Idler I, et al. Targeting inhibitor of apoptosis proteins by Smac mimetic elicits cell death in poor prognostic subgroups of chronic lymphocytic leukemia. Int J Cancer. 2015;137(12):2959-70. Epub 2015/06/23. doi: 10.1002/ijc.29650. PubMed PMID: 26096065.

20. von Knebel Doeberitz M. New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. European journal of cancer (Oxford, England : 1990). 2002;38(17):2229-42. Epub 2002/11/21. PubMed PMID: 12441259.

21. Geng Y, Savage SM, Razani-Boroujerdi S, Sopori ML. Effects of nicotine on the immune response. II. Chronic nicotine treatment induces T cell anergy. J Immunol. 1996;156(7):2384-90. Epub 1996/04/01. PubMed PMID: 8786295.

22. Nakanishi Y, Lu B, Gerard C, Iwasaki A. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. Nature. 2009;462(7272):510-3. Epub 2009/11/10. doi: 10.1038/nature08511. PubMed PMID: 19898495; PubMed Central PMCID: PMCPmc2789415.

23. Toes RE, Ossendorp F, Offringa R, Melief CJ. CD4 T cells and their role in antitumor immune responses. J Exp Med. 1999;189(5):753-6. Epub 1999/03/02. PubMed PMID: 10049938; PubMed Central PMCID: PMCPmc2192956.

24. Holzinger D, Schmitt M, Dyckhoff G, Benner A, Pawlita M, Bosch FX. Viral RNA patterns and high viral load reliably define oropharynx carcinomas with active HPV16 involvement. Cancer Res. 2012;72(19):4993-5003. Epub 2012/09/20. doi: 10.1158/0008-5472.can-11-3934. PubMed PMID: 22991302.

25. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517(7536):576-82. Epub 2015/01/30. doi: 10.1038/nature14129. PubMed PMID: 25631445; PubMed Central PMCID: PMCPmc4311405.

26. Charoentong P, Finotello F, Angelova M, Mayer C, Efremova M, Rieder D, et al. Pan-cancer Immunogenomic Analyses Reveal Genotype-Immunophenotype Relationships and Predictors of Response to Checkpoint Blockade. Cell Rep. 2017;18(1):248-62. Epub 2017/01/05. doi: 10.1016/j.celrep.2016.12.019. PubMed PMID: 28052254.

27. Tang AL, Hauff SJ, Owen JH, Graham MP, Czerwinski MJ, Park JJ, et al. UM-SCC-104: a new human papillomavirus-16-positive cancer stem cell-containing head and neck squamous cell carcinoma cell line. Head Neck. 2012;34(10):1480-91. Epub 2011/12/14. doi: 10.1002/hed.21962. PubMed PMID: 22162267; PubMed Central PMCID: PMCPmc3369005.

28. Brenner JC, Graham MP, Kumar B, Saunders LM, Kupfer R, Lyons RH, et al. Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. Head Neck. 2010;32(4):417-26. Epub 2009/09/18. doi: 10.1002/hed.21198. PubMed PMID: 19760794; PubMed Central PMCID: PMCPmc3292176.

29. Becht E, Giraldo NA, Germain C, de Reynies A, Laurent-Puig P, Zucman-Rossi J, et al. Immune Contexture, Immunoscore, and Malignant Cell Molecular Subgroups for Prognostic and Theranostic Classifications of Cancers. Adv Immunol. 2016;130:95-190. Epub 2016/03/01. doi: 10.1016/bs.ai.2015.12.002. PubMed PMID: 26923001.

30. Braumuller H, Wieder T, Brenner E, Assmann S, Hahn M, Alkhaled M, et al. T-helper-1-cell cytokines drive cancer into senescence. Nature. 2013;494(7437):361-5. Epub 2013/02/05. doi: 10.1038/nature11824. PubMed PMID: 23376950.

31. van der Sluis TC, van Duikeren S, Huppelschoten S, Jordanova ES, Beyranvand Nejad E, Sloots A, et al. Vaccine-induced tumor necrosis factor-producing T cells synergize with cisplatin to promote tumor cell death. Clin Cancer Res. 2015;21(4):781-94. Epub 2014/12/17. doi: 10.1158/1078-0432.ccr-14-2142. PubMed PMID: 25501579.

32. Yang G, Xu Y, Chen X, Hu G. IFITM1 plays an essential role in the antiproliferative action of interferon-gamma. Oncogene. 2007;26(4):594-603. Epub 2006/07/19. doi: 10.1038/sj.onc.1209807. PubMed PMID: 16847454.

33. Wu CC, Tsai FM, Shyu RY, Tsai YM, Wang CH, Jiang SY. G protein-coupled receptor kinase 5 mediates Tazarotene-induced gene 1-induced growth suppression of human colon cancer cells. BMC Cancer. 2011;11:175. Epub 2011/05/18. doi: 10.1186/1471-2407-11-175. PubMed PMID: 21575264; PubMed Central PMCID: PMCPmc3112162.

34. Kang YH, Seigel B, Bengsch B, Fleming VM, Billerbeck E, Simmons R, et al. CD161(+)CD4(+) T cells are enriched in the liver during chronic hepatitis and associated with co-secretion of IL-22 and IFN-gamma. Front Immunol. 2012;3:346. Epub 2012/11/28. doi: 10.3389/fimmu.2012.00346. PubMed PMID: 23181064; PubMed Central PMCID: PMCPmc3502006.

35. Ramos CA, Narala N, Vyas GM, Leen AM, Gerdemann U, Sturgis EM, et al. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes for adoptive immunotherapy of HPV-associated malignancies. J Immunother. 2013;36(1):66-76. Epub 2012/12/06. doi: 10.1097/CJI.0b013e318279652e. PubMed PMID: 23211628; PubMed Central PMCID: PMCPmc3521877.

36. Linnemann C, van Buuren MM, Bies L, Verdegaal EM, Schotte R, Calis JJ, et al. High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. Nat Med. 2015;21(1):81-5. Epub 2014/12/23. doi: 10.1038/nm.3773. PubMed PMID: 25531942.

37. Muranski P, Restifo NP. Essentials of Th17 cell commitment and plasticity. Blood. 2013;121(13):2402-14. Epub 2013/01/18. doi: 10.1182/blood-2012-09-378653. PubMed PMID: 23325835; PubMed Central PMCID: PMCPmc3612853.

38. Balermpas P, Rodel F, Rodel C, Krause M, Linge A, Lohaus F, et al. CD8+ tumour-infiltrating lymphocytes in relation to HPV status and clinical outcome in patients with head and neck cancer after postoperative chemoradiotherapy: A multicentre study of the German cancer consortium radiation oncology group

(DKTK-ROG). Int J Cancer. 2016;138(1):171-81. doi: 10.1002/ijc.29683. PubMed PMID: 26178914.

39. Kindt N, Descamps G, Seminerio I, Bellier J, Lechien JR, Pottier C, et al. Langerhans cell number is a strong and independent prognostic factor for head and neck squamous cell carcinomas. Oral Oncol. 2016;62:1-10. Epub 2016/11/21. doi: 10.1016/j.oraloncology.2016.08.016. PubMed PMID: 27865360.

40. Rajjoub S, Basha SR, Einhorn E, Cohen MC, Marvel DM, Sewell DA. Prognostic significance of tumor-infiltrating lymphocytes in oropharyngeal cancer. Ear Nose Throat J. 2007;86(8):506-11. Epub 2007/10/06. PubMed PMID: 17915676.

41. Wang ZQ, Milne K, Derocher H, Webb JR, Nelson BH, Watson PH. CD103 and Intratumoral Immune Response in Breast Cancer. Clin Cancer Res. 2016;22(24):6290-7. Epub 2016/06/09. doi: 10.1158/1078-0432.ccr-16-0732. PubMed PMID: 27267849.

42. Takahashi T, Dejbakhsh-Jones S, Strober S. Expression of CD161 (NKR-P1A) defines subsets of human CD4 and CD8 T cells with different functional activities. J Immunol. 2006;176(1):211-6. Epub 2005/12/21. PubMed PMID: 16365412.

43. Majchrzak K, Nelson MH, Bailey SR, Bowers JS, Yu XZ, Rubinstein MP, et al. Exploiting IL-17-producing CD4+ and CD8+ T cells to improve cancer immunotherapy in the clinic. Cancer Immunol Immunother. 2016;65(3):247-59. Epub 2016/01/31. doi: 10.1007/s00262-016-1797-6. PubMed PMID: 26825102; PubMed Central PMCID: PMCPmc5115162.

44. Iliopoulou EG, Karamouzis MV, Missitzis I, Ardavanis A, Sotiriadou NN, Baxevanis CN, et al. Increased frequency of CD4+ cells expressing CD161 in cancer patients. Clin Cancer Res. 2006;12(23):6901-9. Epub 2006/12/06. doi: 10.1158/1078-0432.ccr-06-0977. PubMed PMID: 17145807.

45. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat Med. 2015;21(8):938-45. doi: 10.1038/nm.3909. PubMed PMID: 26193342.

46. Pignon JP, Bourhis J, Domenge C, Designe L. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer. Lancet. 2000;355(9208):949-55. Epub 2000/04/18. PubMed PMID: 10768432.

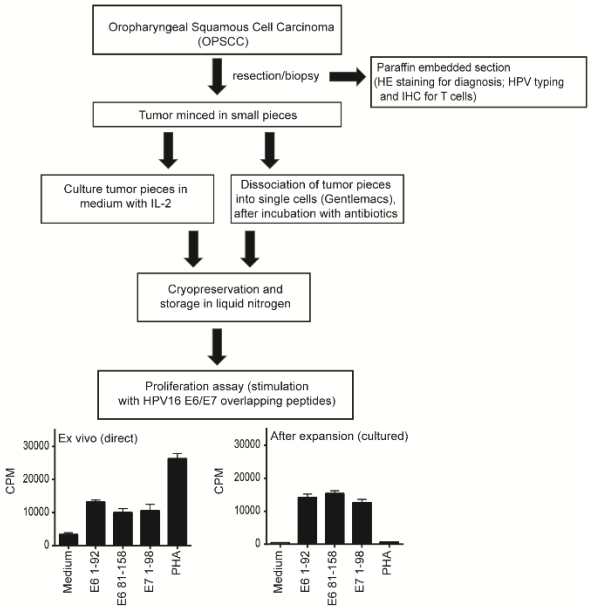
47. Hoek J, Bloemendal KM, van der Velden LA, van Diessen JN, van Werkhoven E, Klop WM, et al. Nephrotoxicity as a Dose-Limiting Factor in a High-Dose Cisplatin-Based Chemoradiotherapy Regimen for Head and Neck Carcinomas. Cancers (Basel). 2016;8(2). Epub 2016/02/20. doi: 10.3390/cancers8020021. PubMed PMID: 26891330; PubMed Central PMCID: PMCPmc4773744.

48. Rades D, Fehlauer F, Sheikh-Sarraf M, Kazic N, Basic H, Poorter R, et al. Toxicity of two cisplatin-based radiochemotherapy regimens for the treatment of patients with stage III/IV head and neck cancer. Head Neck. 2008;30(2):235-41. Epub 2007/07/28. doi: 10.1002/hed.20683. PubMed PMID: 17657790.

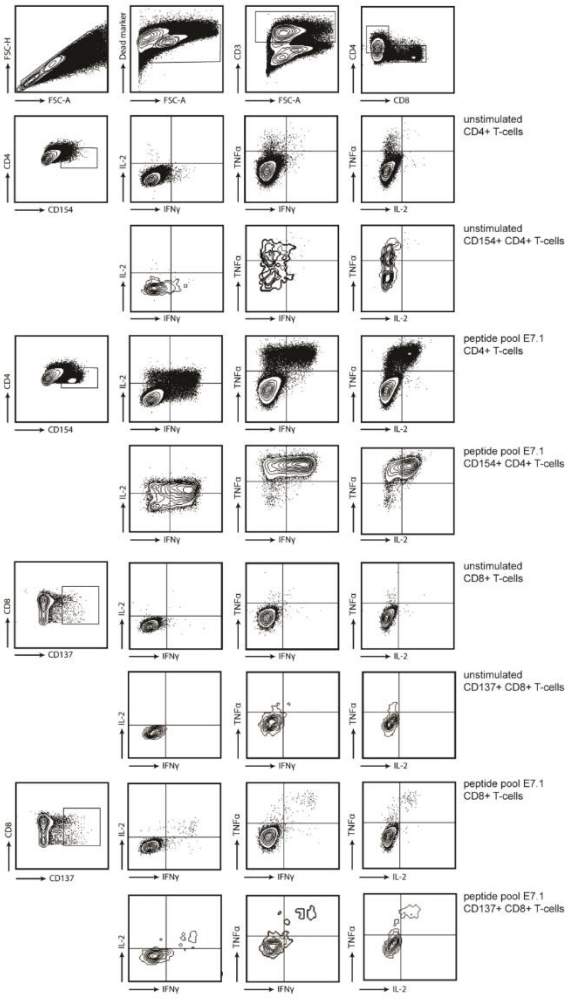
49. Blank CU, Haanen JB, Ribas A, Schumacher TN. CANCER IMMUNOLOGY. The "cancer immunogram". Science. 2016;352(6286):658-60. Epub 2016/05/07. doi: 10.1126/science.aaf2834. PubMed PMID: 27151852.

50. Lyford-Pike S, Peng S, Young GD, Taube JM, Westra WH, Akpeng B, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res. 2013;73(6):1733-41. Epub 2013/01/05. doi: 10.1158/0008-5472.can-12-2384. PubMed PMID: 23288508; PubMed Central PMCID: PMCPmc3602406.

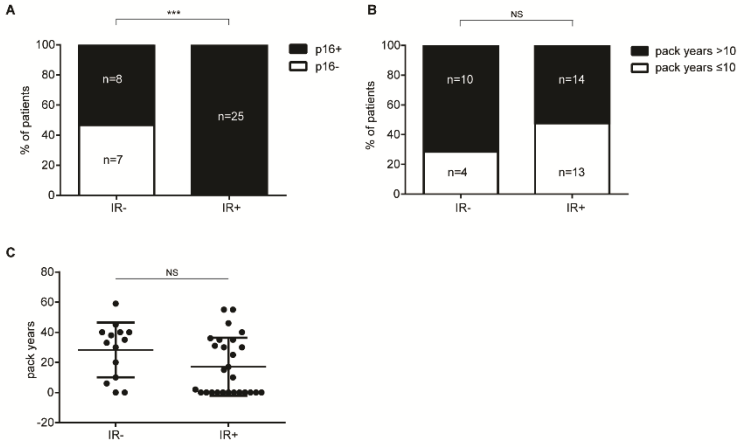
SUPPLEMENTARY INFORMATION



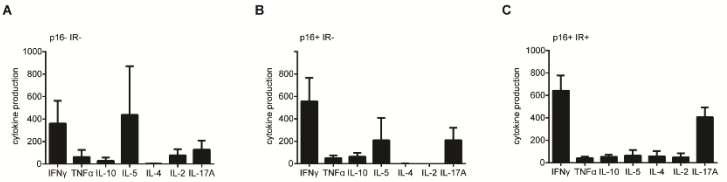
Supplementary Figure S1. Handling of oropharyngeal squamous cell carcinoma (OPSCC) material. Schematic overview of the handling of the tumors from OPSCC patients obtained either via resection or biopsy in the operation theater or via biopsy taken in the out-patient clinic. For diagnosis tumor material is embedded in paraffin to perform HE staining. In these paraffin embedded tissues also sections were made to perform HPV typing and $p16^{INK4a}$ as well as immunofluorescent double staining. In parallel tumor pieces were minced in small pieces and either mechanically dissociated using the Gentlemacs and/or put in culture in medium with addition of IL-2. The TILs were cryopreserved after the dissociation and upon sufficient number of cells after culture stored in the vapour phase of liquid nitrogen until use. Thawed TILs were subjected to a proliferation assay using autologous monocytes loaded with pools of peptides covering the HPV oncoproteins E6 and E7. The proliferation is depicted as counts per minutes (CPM). Cells in medium only served as negative control and PHA was taken along as a positive control.



Supplementary Figure S2. HPV16-positive OPSCC harbor T-cells that specifically recognize the HPV16 oncoproteins E6 and E7. A representative example of the intracellular cytokine staining (ICS) performed on the cultured TILs from HPV16-positive OPSCC patient is given. The gating strategy is shown in which the living single cells were further gated for viable cells and then CD3 was selected. Within CD3+ cells the CD4 was plotted versus CD8 followed by the CD4 or CD8 plotted versus the activation markers CD154 and CD137, respectively. Either within the CD4 or CD8 population or within the activated T-cells the different combinations of the cytokines IFNγ, IL-2 and TNFα produced were depicted. Shown is the unstimulated TILs and the condition in which the TILs were stimulated overnight with peptide pool E7.1 of HPV16.



Supplementary Figure S3. High expression of HPV16 antigens by HPV16-positive OPSCC results in induction of HPV16-specific T-cells. **A,** From 40 of the 45 HPV16-positive OPSCC patients tested for a HPV-specific immune response the $p16^{INK4a}$ expression was determined. The patients were grouped according to these two parameters. All patients with an immune response to HPV16 (IR+) were also $p16^{INK4a}$ positive, while in the IR- group 7 patients had a rather low expression of the HPV16 oncoproteins on the tumor cells revealing a negative score for $p16^{INK4a}$. The frequencies and number of patients are depicted for IR- and IR+ as well as the positive and negative score of $p16^{INK4a}$. **B,** The patients were divided on basis of the pack years (>10 years versus ≤ 10 years), which was available for 41 of the 45 tested HPV16-positive OPSCC patients. Fisher Exact analysis was performed for the data displayed in **A** and **B**. **C,** The pack years are given for the individual HPV16-positive OPSCC patients and shown in the IR- versus IR+ group as in **A** and **B**. An almost significant difference between the two groups of patients was observed ($P = 0.053$). NS, not significant.



Supplementary Figure S4. PHA stimulation of TILs results in differential cytokine production in the various groups of OPSCC patients. The overall cytokine production upon PHA stimulation was determined at the same time as the HPV16-specific responses in the 5-day proliferation assay. Supernatant at day 4 was subjected to cytometric bead array (CBA) analysis to determine the production of IFNγ, TNFα, IL-10, IL-5, IL-4 and IL-2 and is depicted as pg/mL (± SEM). IL-17A is determined by ELISA. The results are shown for the three groups of the OPSCC patients: **A,** p16-IR- (n=5), **B,** p16+IR- (n=4) and **C,** p16+IR+ (n=17).

Chapter 5

EGFR signaling suppresses type 1 cytokine-induced T-cell attracting chemokine secretion in head and neck cancer.

Ma W, Concha-Benavente F, Santegoets SJ, Ehsan I, Welters MJP, Ferris RL, Van Der Burg SH

PLoS One. 2018 Sep 7;13(9):e0203402

EGFR signaling suppresses type 1 cytokine-induced T-cell attracting chemokine secretion in head and neck cancer.

Wenbo Ma[1], Fernando Concha-Benavente[2], Saskia J.A.M. Santegoets[1], Marij J.P. Welters[1], Ilina Ehsan[1], Robert L. Ferris[2], Sjoerd H. van der Burg[1,§].

[1]Department of Medical Oncology, Leiden University Medical Center, Leiden, The Netherlands.

[2]Department of Otolaryngology, University of Pittsburgh, and the University of Pittsburgh Cancer Institute, Pittsburgh, PA, United States

[§]Corresponding author: Prof. Dr. S.H. van der Burg, Dept. of Medical Oncology, Building 1, C7-147, Leiden University Medical Center, PO box 9600, 2300 RC Leiden, The Netherlands. Phone: +31-71-5261180, Fax: +31-71-5266760, E-mail: shvdburg@lumc.nl

Abstract

Objectives:
Resistance to antitumor immunity can be promoted by the oncogenic pathways operational in human cancers, including the epidermal growth factor receptor (EGFR) pathway. Here we studied if and how EGFR downstream signaling in head and neck squamous cell carcinoma (HNSCC) can affect the attraction of immune cells.

Material & Methods:
HPV-negative and HPV-positive HNSCC cell lines were analyzed in vitro for CCL2, CCL5, CXCL9, CXCL10, IL-6 and IL-1β expression and the attraction of T cells under different conditions, including cetuximab treatment and stimulation with IFNγ and TNFα using qPCR, ELISA and migration experiments. Biochemical analyses with chemical inhibitors and siRNA transfection were used to pinpoint the underlying mechanisms.

Results:
Stimulation of HNSCC cells with IFNγ and TNFα triggered the production of T-cell attracting chemokines and required c-RAF activation. Blocking of the EGFR with cetuximab during this stimulation increased chemokine production in vitro, and augmented the attraction of T cells. Mechanistically, cetuximab decreased the phosphorylation of MEK1, ERK1/2, AKT, mTOR, JNK, p38 and ERK5. Chemical inhibition of EGFR signaling showed a consistent and pronounced chemokine production with MEK1/2 inhibitor PD98059 and JNK inhibitor SP600125, but not with inhibitors of p38, PI3K or mTOR. Combination treatment with cetuximab and a MEK1/2 or JNK inhibitor induced the highest chemokine expression.

Conclusion:
Overexpression of EGFR results in the activation of multiple downstream signaling pathways that act simultaneously to suppress type 1 cytokine stimulated production of chemokines required to amplify the attraction of T cells.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide. The risk factors of HNSCC are tobacco and alcohol use or an infection with high-risk human papillomaviruses, in particular type 16 (HPV16) [1]. Previous studies have shown that patients with strongly T-cell infiltrated HNSCC display better survival [2-4]. Interestingly, the group of patients with an HPV-induced HNSCC have a much better prognosis than their HPV-negative counterparts [5] and this was related to heavy tumor-infiltration by activated CD4+ and CD8+ T cells [6-8]. Importantly, a prospective study on the role of tumor-specific T-cell responses in HNSCC showed that the viral antigens in HPV16+ HNSCC triggered an intratumoral IFNγ- and TNFα-producing HPV-specific T cell response which shaped a favorable type 1 immune contexture and was strongly associated with a good clinical response to standard (chemo)radiotherapy [9]. HNSCC patients that are refractory to first line therapy may respond upon treatment with an anti-programmed cell death protein-1(PD-1) monoclonal antibody [10]. Together, these data reveal that the immune response play an important role in HNSCC but also that in many cases such a response is lacking.

The epidermal growth factor receptor (EGFR) is frequently overexpressed in cancers of patients with poor prognosis and is found in 80-90% of HNSCC [11]. EGFR overexpression results in increased cell proliferation, cell migration and resistance to apoptosis. Based on this a number of agents targeting EGFR have been developed and are now used to treat HNSCC patients, one of which is the monoclonal antibody cetuximab [12]. Cetuximab blockade of EGFR will inhibit tumor growth, DNA damage repair, and metastasis. It does so by interfering with the binding of the natural ligands to EGFR as well as by inducing receptor endocytosis, thereby disrupting EGFR signaling. Furthermore, cetuximab may also trigger antibody dependent cell-mediated cytotoxicity [13] More recent data, however, suggest that activation of EGFR signaling itself also bears an immune regulatory component. EGFR activation represses MHC class I and II expression [14] as well as promotes the expression of PD-L1 in lung cancers and

HNSCC [15, 16]. Furthermore, the presence of an EGFR mutation is related to lower T-cell infiltration [17]. The downstream targets of EGFR include the Raf-MEK-ERK pathway, the MAP3K pathway and the PI3K-AKT-mTOR pathway [18]. Interestingly, loss of PTEN resulted in decreased T-cell infiltration and resistance to PD-1 blocking in preclinical models of melanoma. T cell infiltration and tumor control could be restored by blocking PI3K-AKT signaling using the inhibitor PI3Kβ [19]. These data suggest that the EGFR downstream signaling pathways may, similar to other recently reported oncogenic pathways [20, 21], attenuate tumor immunity by preventing T cell infiltration.

In order to study if and how EGFR downstream signaling may affect immune infiltration, we made use of HPV- and HPV+ HNSCC cell lines which were stimulated with type 1 cytokines in the absence and presence of cetuximab or inhibitors of molecules downstream of the EGFR. We show that blocking EGFR with cetuximab inhibits the activation of several pathways downstream of EGFR and results in an increased production of inflammatory chemokines and attraction of T cells when the tumor cells are stimulated with IFNγ and TNFα. Mechanistically, EGFR signaling suppressed type 1 cytokine-induced chemokine production in a MEK and JNK dependent fashion.

Material and Methods

Cell culture

The HNSCC cell lines were obtained from the University of Michigan (Ann Arbor, MI, USA) and designated as UM-SCC. We obtained UM-SCC4 and UM-SCC19 (both HPV negative) as well as UM-SCC47 and UM-SCC104 (both HPV16-positive) in 2012. The cells were cultured in RPMI 1640 (Gibco/ Thermo Fisher Scientific (TFS) Bleiswijk, the Netherlands) with 10% Fetal Calf Serum (PAA laboratories; Pashing, Austria) and penicillin/streptomycin (TFS). Microsatellite analysis was performed in July 2016 by BaseClear (Leiden, the Netherlands) to assure cell line authentication when the experiments were performed. Mycoplasma was tested on a monthly basis and found negative.

Patients and specimens

All patients signed an informed consent approved by the Institutional Review Board (IRB #99-06) of the University of Pittsburgh Cancer Institute. Peripheral venous blood samples were obtained from HNC patients with stage III/IVA disease, receiving neoadjuvant single-agent cetuximab on a prospective phase II clinical trial (UPCI 08-013, NCT 01218048). Serum and plasma specimens were isolated from blood specimens and stored in aliquots frozen at -80 Celsius until cytokine determination. A representative sample of plasma/serum specimens from 20 patients was selected for cytokine determination. Demographics: 12 patients were randomly with age ranging from 49 to 93 years old. Samples of frozen serum specimens were thawed at room temperature for 15 minutes before starting the protocols. Human CXCL-9 (cat#DCX900, Sensitivity: 11.3 pg/mL) ELISA quantikine kits (R&D systems, Minneapolis, MN) and Human CXCL10 (cat# HCYTMAG-60K-PX29) Milliplex kit (Millipore, Minneapolis, MN) were determined according to the manufacturers protocol.

Reagents

Recombinant human TNFα (Rhtnf-a, Invivogen/bioconnect,France), Recombinant Human Interferon-γ (11343536, Immunotools, Germany). Cetuximab (5 mg/ml; Merck serono, USA), rituximab (10 mg/ml; Roche, Switzerland), rapamycin (50 nM; Selleckchem,

Germany), PD98059 (50 μM; Selleckchem), GW5074 (20 μM; Selleckchem), LY294002(25 μM; Selleckchem),SP600125 (20 μM; Selleckchem). Pamapimod (0,5 μM Selleckchem), JSH-23(10 μM, Selleckchem), and T-5224(20μM, Apexbt, USA). The concentration of GW5074, SP600125, LY-294,002, PD98059, Rapamycin was chosen on basis of previous results [22]. The concentration of Pamapimod (0,5uM Selleckchem), JSH-23(10μM, Selleckchem), and T-5224(20μM, Apexbt) was according to instruction of the manufacturer.

RNA expression analysis

Total RNA was isolated using the RNeasy Plus Mini Kit (74134 Qiagen) according to the manufacturer's instructions. Total RNA (0.5–1.0 μg) was reverse transcribed using the SuperScript III First Strand synthesis system from Invitrogen. TaqMan PCR was performed (each sample in triplicates) using TaqMan Universal PCR Master Mix and pre-designed, pre-optimized primers and probe mix for CCL2, CCL5, CXCL9, CXCL10, IL6, IL1β and GAPDH (Applied Biosystems, Foster City, USA). Threshold cycle numbers (Ct) were determined using the CFX PCR System (Bio-Rad, Veenendaal, The Netherlands), and the relative quantities of cDNA per sample were calculated using the ΔΔCt method using GAPDH as the calibrator gene.

Western blot analysis

Polypeptides were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Veenendaal, The Netherlands). Immune detection was achieved with primary antibodies against Phospho-c-Raf (Ser338, 9427T), Phospho-MEK1/2 (Ser217/221, 9154T), Phospho-ERK1/2 (Thr202/Tyr204, 4370T), Phospho-AKT (Ser473, 4060T), Phospho-mTOR (Ser2448, 5536T), Phospho-SAPK/JNK (Thr183/Tyr185, 4668T), Phospho-p38 MAPK (Thr180/Tyr182, 4511T), Phospho-Erk5 (Thr218/Tyr220, 3371s), IRF-1(8478s) , IRF-3(11904), anti- anti-acetyl-p65 (Lys310,3045), anti-phospho-p65 (Ser536, 3033), STAT1 (#9172, CST), phospho-STAT1 (Tyr701, #9167) HRP-coupled anti-mouse (#7076s) and HRP-coupled anti-rabbit (#7074s) secondary antibodies were purchased from CST, USA. IFRD1(T2576) and β-actin (A5316) were purchased from Sigma-Aldrich. Chemoluminescence

reagent (#170-5060, Bio-Rad) was used as substrate and signal was scanned using the Chemidoc and accompanying Software (Image Lab Software Version 5.2.1, Bio-Rad).

Enzyme-linked immunosorbent assays (ELISAs) on cell supernatants
CXCL9 and CXCL10 were performed according to the manufacturer's instruction (PeproTech,
London, UK).

Chemotaxis assays
The assay for chemotaxis was performed in 24-well plates (Costar, Cambridge, MA) carrying 6.5 mm Trans-well with 3.0 µm pore polyester membrane insert (CLS3472, Sigma). CD14-depleted PBMC were washed once and suspended at $20*10^6$ cells/ml in serum free RPMI 1640 medium. Supernatant were placed in the lower compartment, and cells were loaded onto the inserts at $2*10^6$/100µL each individual assay. Chambers were incubated for 4h in a 5% CO2-humidified incubator at 37°C. After the incubation period, numbers of CD14-depleted PBMC migrating to the lower chamber were counted by flow cytometry (BD Accuri™ C6) using counting Beads (C36950, Thermofisher) and analyzed using FlowJo v10.0.8 (Treestar, Olten, Switzerland). All conditions were tested in triplicate. Statistic evaluation was performed using the Student t test.

Flow cytometry methods for EGFR expression
Expression of EGFR was analyzed by flow cytometry using phycoerythrin (PE)-coupled mouse-anti-human EGFR (1:20, BD Biosciences, Breda, The Netherlands). Per live gate, 50,000 cells were recorded using the BD FACS Calibur with Cellquest software (BD Bioscience) and data were analyzed using Flowjo.

Ethics Statement
This study is part of a larger study P07-112, approved by the local medical ethical committee of the Leiden University Medical Center (LUMC) and in agreement with the Dutch law.

Results

EGFR signaling suppresses IFNγ/TNFα-mediated production of immune cell attracting chemokines.

In order to test the effects of EGFR signaling on T cell attraction, we used the two HPV-negative UM-SCC4 and UM-SCC19 and the two HPV-positive HNSCC cell lines UM-SCC47 and UM-SCC104 for the expression of CCL2, CCL5, CXCL9, CXCL10, IL-6 and IL-1β by qPCR. All 4 cell lines display high EGFR expression (Supplementary Fig. 1) and were able to signal via their IFNγ and TNFα receptors as shown by us before [9]. The cells were stimulated with or without a combination of IFNγ and TNFα as well as pre-treated with either the EGFR blocking antibody cetuximab or as control rituximab, a monoclonal antibody against CD20, for 48 hours as this will allow for functional effects of EGFR blockade on downstream targets without causing overt effects on cell density [22]. While both IFNγ and TNFα are able to increase the expression of certain chemokine genes, their combination results in even higher gene expression (Supplementary Fig. 2). Treatment with the EGFR blocking antibody cetuximab alone didn't alter the expression of cytokines. However, when the tumor cells were also stimulated with the type 1 cytokine IFNγ and TNFα, an increased expression of *CCL2, CCL5, CXCL9, CXCL10* and *IL-6* was detected when compared to treatment with the control antibody (Fig. 1A). In addition, cetuximab led to the decreased expression of *IL-1*β (Fig. 1A). Several of the chemokines produced by the tumor cells, including CXCL9 and CXCL10, are important with respect to their capacity to attract T cells. Analysis of the supernatants isolated from the cultures of treated cells showed an increased amount of cytokines produced when the EGFR was blocked (Fig. 1B & Supplementary Fig. 3A). Interestingly, the levels of these two chemokines were also found to be increased in many patients with head and neck cancers after treatment with cetuximab (Supplementary Fig. 3B). In addition, the tumor cell supernatant was used to study lymphocyte migration. In all 4 cases, enhanced lymphocyte infiltration was observed when PBMC where incubated with tumor cell supernatant of cetuximab treated IFNγ/TNFα stimulated cancer cells (Fig. 1C). This included both CD4+ and CD8+ lymphocytes (Supplementary Fig. 4). In

conclusion, EGFR signaling in squamous cell carcinoma cells from the head and neck represses the production of several IFNγ/TNFα-induced T-cell attracting chemokines.

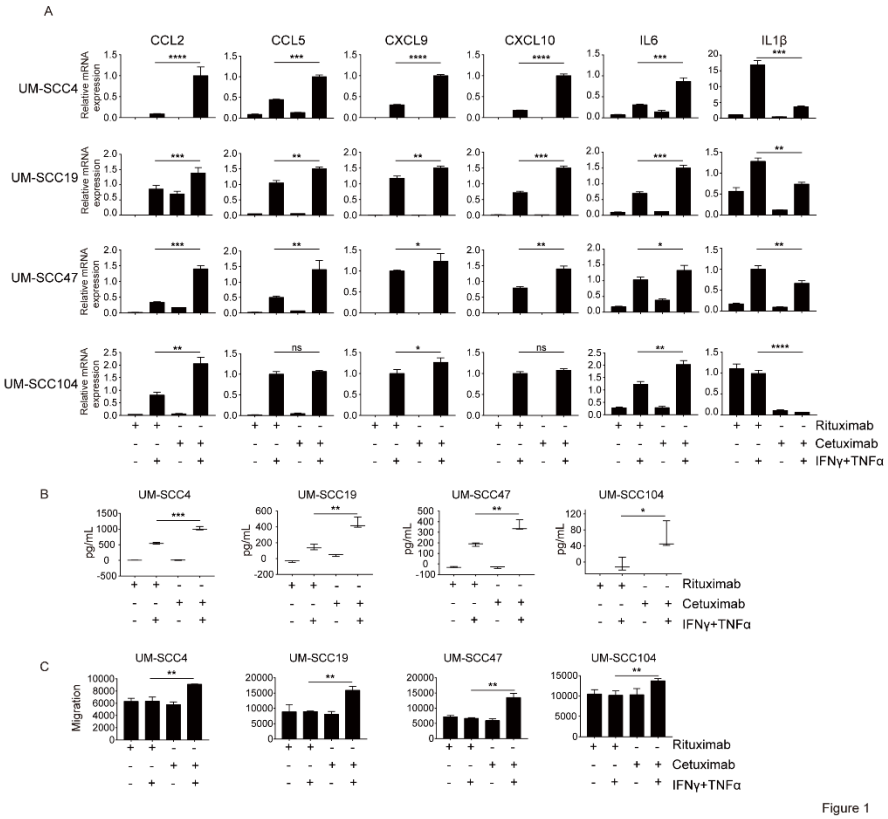


Figure 1 EGFR signaling suppresses IFNγ/TNFα-mediated production of immune cell attracting chemokines

Two HPV- HNC cell lines (UM-SCC4 and UM-SCC19) and two HPV+ HNC cell line (UM-SCC47 and UM-SCC104) were stimulated with 1µg/mL rituximab or 1µg/mL cetuximab as indicated. After 48h, the cells were treated with 50IU/mL IFNγ and 30ng/mL TNFα. (A)After 24h, the expression levels of the genes indicated were determined by RT-qPCR. Gene expression was normalized against GAPDH mRNA levels. Similar results were observed in two independent experiments. (B) After 48h the concentration of CXCL9 was determined by Enzyme-

linked immunosorbent assay in supernatants of three different experiments. (C) The supernatant isolated after 48h were used to study CD14-depleted PBMC migration in transwell assays. P value were determined by unpaired t-tests. Ns: no significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Cetuximab blocks the activation of several downstream EGFR signaling pathways.

In order to understand the mechanism underlying the repression of chemokine production by EGFR signaling, an analysis of the downstream EGFR signaling pathways was executed. The phosphorylation of the proteins in the Raf-MEK-ERK pathway, the PI3K-AKT-mTOR pathway and the MAP3K pathway was analyzed. Cetuximab treatment led to the reduction of Raf-MEK-ERK pathway and PI3K-AKT-mTOR pathway in UM-SCC4, UM-SCC47 and UM-SCC104, but not in UM-SCC19. In addition, phosphorylation of p38 and ERK5 was decreased in all cell lines upon cetuximab treatment (Fig. 2).

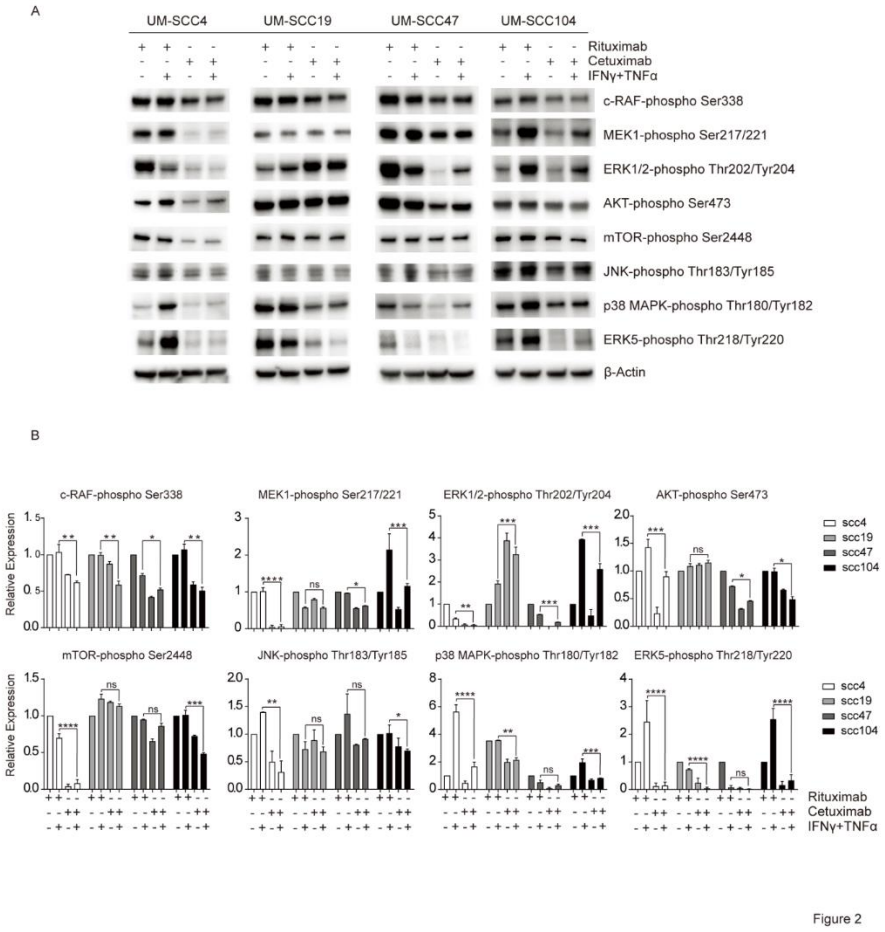


Figure 2

Figure 2 Cetuximab blocks the activation of several downstream EGFR signaling pathways

(A) Two HPV- HNC cell lines (UM-SCC4 and UM-SCC19) and two HPV+ HNC cell line (UM-SCC47 and UM-SCC104) were stimulated with 1μg/mL rituximab or 1μg/mL cetuximab as indicated. After 48h, the cells were treated with 50IU/mL IFNγ and 30ng/ml TNFα for 24h. The protein expression levels of the phosphorylated proteins were measured by Western blotting in whole cell extracts. β-actin served as loading control. (B)Relative density of proteins were quantified over β-actin. The expression levels of rituximab treatment were set as 1. Similar results were observed in two independent experiments. P

value were determined by unpaired t-tests. Ns: no significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Previously, we had found that EGFR blocking by cetuximab may also lead to the reduction of IRFD1. IRFD1 blocks enhanced cytokine production by recruiting HDAC1/3 in order to prevent acetylation of to the nucleus translocated phosphorylated p65 [22]. Therefore, also the expression and activation of multiple transcription factors was analyzed. Indeed, EGFR blocking decreased IFRD1 expression in the cell lines UM-SCC4 and UM-SCC104, but only in UM-SCC104 this resulted in increased p65 acetylation. The lack of an effect on UM-SCC4 was expected as it also failed to show p65 phosphorylation (Supplemental Fig. 5). In UM-SCC47, cetuximab treatment resulted in increased STAT1 nuclear translocation and increased phosphorylation of p65 (Supplemental Fig. 5) but this was not found in the other cell lines.

Interferon regulatory factors (IRF), in particular IRF1 and IRF3, have been found to regulate chemokine production [23, 24]. Therefore, IRF1 and IRF3 were knocked-down in UM-SCC4 and UM-SCC47. This showed that *CXCL10* but not *CXCL9* expression was dependent on the IRF3 in both cell lines and partly on IRF1 in UM-SCC47 (Supplemental Fig. 6). No effects were seen on the expression of the other cytokines. Furthermore, cetuximab plus IFNγ/TNFα treatment of the cells had no major effects on the expression of IRF1 and IRF3 or phosphorylation of STAT1 (Supplemental Fig. 5). In addition, knock-down of p65 affected the expression of *CXCL10* but had no consistent effect on the expression of the other cytokines. (Supplemental Fig. 6). Moreover, we used the NF B-inhibitor JSH-23 and AP1-inhibitor T-5224 but this did not have an impact on the gene expression of the 3 tested chemokines (Supplemental Fig. 7A). These data suggests that the increased levels of cytokines, in the squamous cell carcinoma cells from the head and neck, are induced by cetuximab plus IFNγ/TNFα independent of the NFκB and AP1 pathways.

Overall, these data show that blocking of the EGFR affects its downstream signaling pathways, albeit differently in individual cell lines. Furthermore, the positive effects on chemokine production observed in all cell lines could not be attributed to the down- or up-regulation of one particular signaling pathway or transcription factor.

The MEK and JNK pathways downstream of EGFR repress chemokine expression.

To mimic the effects of EGFR blocking, several chemical inhibitors of the molecules in the pathways downstream of EGFR were used followed by stimulation with IFNγ/TNFα (Fig. 3). Each of the cell lines responded individually to these inhibitors but the most pronounced effects were seen when the MEK1/2 inhibitor PD98059 and the JNK inhibitor SP600125 were used. In the HPV-negative HNC cell lines UM-SCC4 and UM-SCC19, MEK1 and JNK inhibition led to the most pronounced increase in chemokine expression. In the HPV-positive HNC cell lines UM-SCC47 and UM-SCC104 blocking of JNK signaling resulted in the strongest increase of chemokine gene expression (Fig. 3). The results of PI3K and mTOR inhibition were more variable between the cell lines and less pronounced when compared to MEK and JNK inhibition (Fig. 3), despite the strong inhibition of downstream activation (Supplemental Fig. 8). P38-signaling is known to regulate cytokine production [25], but the use of the p38 inhibitor pamapimod did not affect chemokine expression when the cells were stimulated with IFNγ/TNFα (Supplemental Fig. 7B). Thus, the EGFR-mediated suppression of IFNγ/TNFα induced chemokine expression in squamous cell carcinoma cells from the head and neck primarily is mediated by both the MEK and JNK signaling pathways.

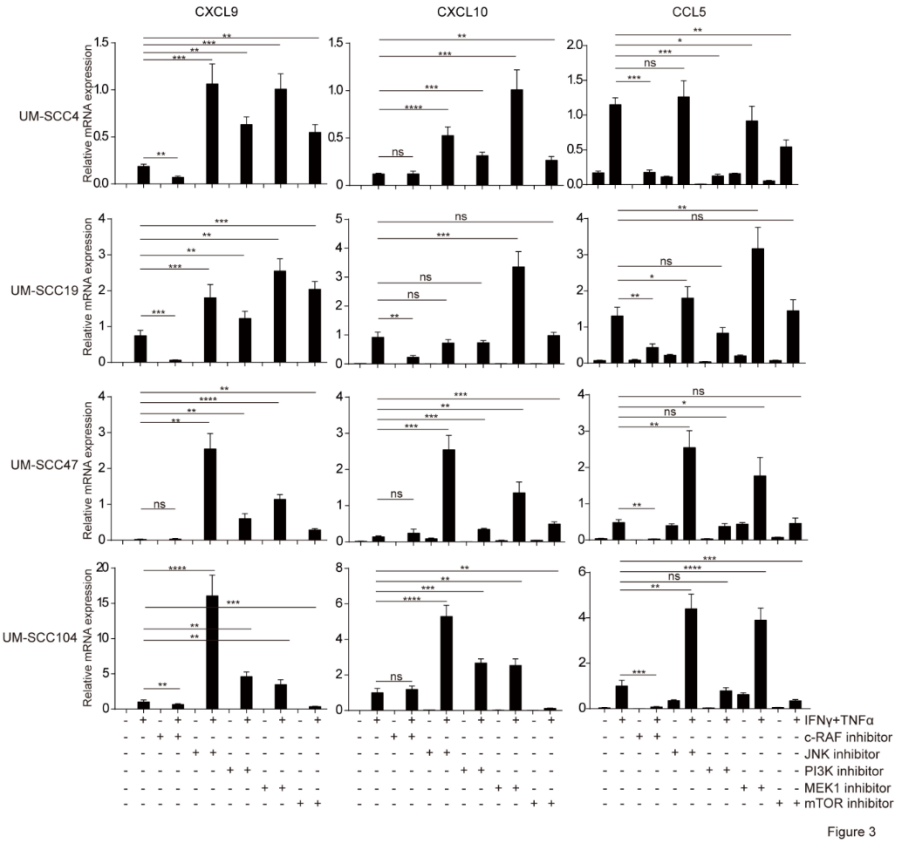


Figure 3 The MEK and JNK pathways downstream of EGFR repress chemokine expression

Two HPV- HNC cell lines (UM-SCC4 and UM-SCC19) and two HPV+ HNC cell line (UM-SCC47 and UM-SCC104) were stimulated with 20μM GW5074 (c-RAF inhibitor), 20μM SP600125 (JNK inhibitor), 25μM LY-294,002 (PI3K inhibitor), 50μM PD98059 (MEK1 inhibitor), 50nM Rapamycin (mTOR inhibitor) as indicated. After 48h, the cells were treated with 50IU/mL IFNγ and 30ng/ml TNFα as indicated for 24h, the expression levels of the genes indicated were determined by RT-qPCR. Gene expression was normalized against GAPDH mRNA levels. Similar results were observed in two independent experiments. P value were determined by unpaired t-tests. Ns: no significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

IFNγ/TNFα-induced chemokine production is c-RAF dependent

Following EGFR signaling the RAF protein kinases become activated and translate the signal to the downstream MEK and MAP3K pathways, hence it would be logical to block this upstream target. EGFR blockade by cetuximab induced only a slightly decrease of c-RAF phosphorylation (Fig. 2). However, treatment of the cell lines with the c-RAF inhibitor GW5074 resulted in a reduced expression of *CCL5*, *CXCL9* and *CXCL10* following IFNγ/TNFα stimulation in all cell lines and this negative effect could not be rescued by co-treatment of the cells with cetuximab (Fig. 4). Potentially, this inhibition is related to the increased phosphorylation of ERK1/2 and MEK1 observed when the c-RAF inhibitor GW5074 was used (Supplemental Fig. 8E). Interestingly, blocking of c-RAF by GW5074 increased the expression levels of *IL-1*β, an effect that was partly reduced by cetuximab (Fig. 4). These data suggest that c-RAF signaling is a dominant factor in the IFNγ/TNFα-mediated induction of chemokine expression in the HNC cell lines. Our observations are sustained by other studies showing that activation of the IFNγR and the TNFR can result in the activation of RAF [26, 27].

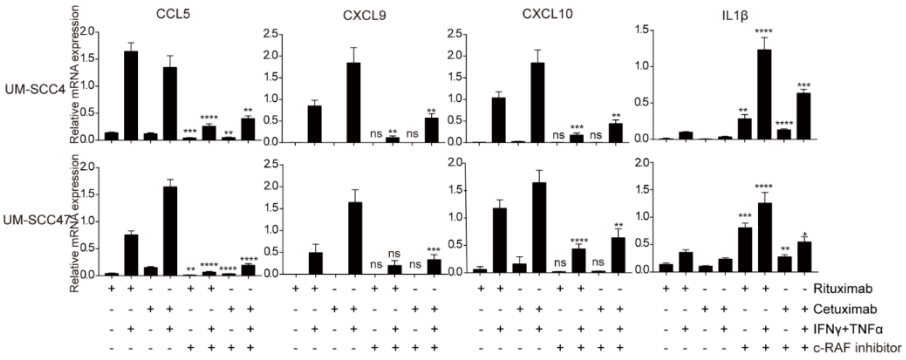


Figure 4

Figure 4 IFNγ/TNFα-induced chemokine production depends on c-RAF signalling and is not blocked by cetuximab

The HPV- HNC cell line UM-SCC4 and the HPV+ HNC cell line UM-SCC47 were stimulated with 1µg/mL rituximab or 1µg/mL cetuximab as indicated for 72h, 20 µM GW5074 (c-RAF inhibitor) as indicated for 48h, 50IU/mL IFNγ and 30ng/ml TNFα as indicated for 24h. The expression levels of the genes indicated were determined by RT-qPCR. Gene expression was normalized against GAPDH mRNA levels. Similar results were observed in two independent experiments. P value were determined by unpaired t-tests. Ns: no significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

S*ynergistic effects of MEK and JNK inhibition with cetuximab shows that different signaling pathways act simultaneously.*

In order to test if blocking of MEK1, JNK or mTOR downstream of EGFR would mimic the results obtained when treating the cells with cetuximab, the tumor cells were incubated with cetuximab and/or one of the chemical inhibitors PD98059 (MEK), SP600125 (JNK) and Rapamycin (mTOR) and then stimulated with IFNγ/TNFα. Blocking of MEK1 or JNK resulted in an enhanced expression of all three chemokines to a level that was similar or higher to those seen when cetuximab alone was used (Fig. 5A-B).

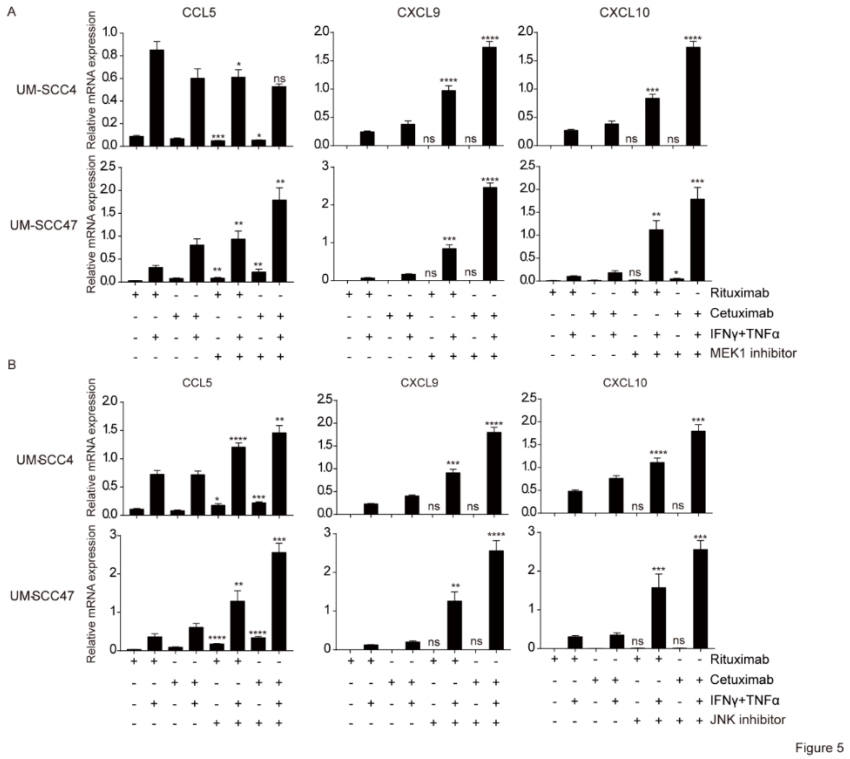


Figure 5 Synergistic effects of MEK and JNK inhibition with cetuximab shows that different signalling pathways act simultaneously.

The HPV- HNC cell line UM-SCC4 and the HPV+ HNC cell line UM-SCC47 were stimulated with 1µg/mL rituximab or 1µg/mL cetuximab as indicated for 72h, a specific inhibitor as indicated for 48h, 50IU/mL IFNγ and 30ng/ml TNFα as indicated for 24h. The expression levels of the genes indicated were determined by RT-qPCR. Gene expression was normalized against GAPDH mRNA levels. (A)50µM of PD98059 (MEK1 inhibitor) was used. (B)20µM of SP600125 (JNK inhibitor) was used. Similar results were observed in two independent experiments. P value were determined by unpaired t-tests. Ns: no significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

When the cells were treated with a combination of cetuximab and MEK or JNK, the expression levels of *CCL5, CXCL9* and *CXCL10* increased, confirming that not one but multiple pathways downstream of the EGFR act in concert to block IFNγ/TNFα -induced chemokine expression by these cancer cell lines. At the protein level, JNK inhibition of IFNγ/TNFα stimulated UM-SCC4 and UM-SCC47 cells resulted in an increased secretion of CXCL9 to a level that was not increased by additional EGFR blocking using cetuximab (Fig. 6).

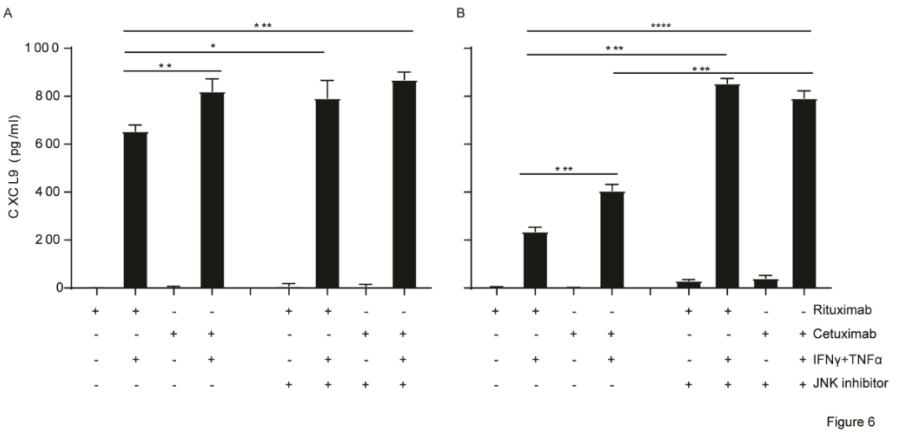


Figure 6 JNK inhibition increases the production of CXCL9 in IFNγ/TNFα-stimulated tumor cells.

The cell lines A) UM-SCC4 and B) UM-SCC47 were stimulated with 1µg/mL rituximab or 1µg/mL cetuximab as indicated for 72h, and the JNK specific inhibitor SP600125 as indicated for 48h, 50IU/mL IFNγ and 30ng/ml TNFα as indicated for 24h. After 48h the concentration of CXCL9 was determined by Enzyme-linked immunosorbent assay in the supernatants P value were determined by unpaired t-tests. Ns: no significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Notably, blocking of mTOR again had variable effects and failed to recapitulate the effect of cetuximab on *CXCL9*. In addition, the levels of the chemokines did not always increase when the cells were treated with cetuximab and the mTOR inhibitor when compared to

treatment with cetuximab only (Supplemental Fig. 7C). In conclusion, overexpression of EGFR results in the activation of the JNK and MEK signaling pathways that act simultaneously to downregulate the IFNγ/TNFα production of chemokines required to attract T cells (Fig. 7).

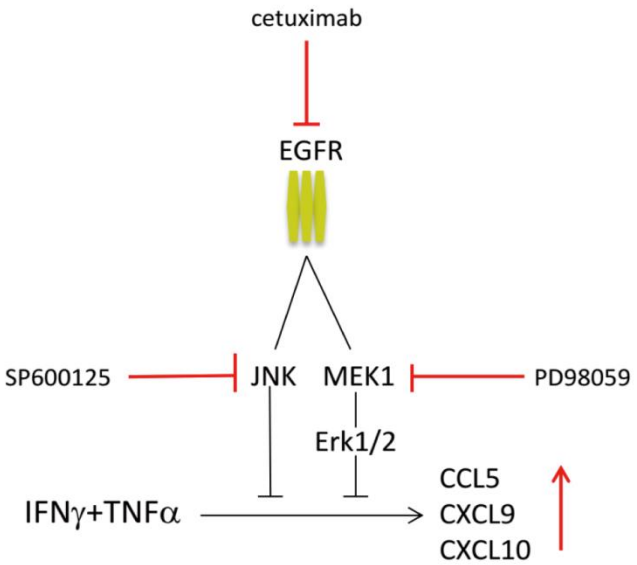


Figure 7 Schematic representation of EGFR mediated repression of chemokine production.

EGFR signalling suppresses IFNγ/TNFα related production of T cell attracting cytokines, including CCL5, CXCL9 and CXCL10 mainly through activation of JNK and MEK1 signaling. Inhibition of EGFR signaling with cetuximab, JNK inhibitor SP600125 and/or MEK1 inhibitor PD98059, restores the IFNγ/TNFα stimulated production of T cell attracting cytokines.

Discussion

In this study, we analyzed the immune regulatory properties of EGFR signaling in head and neck cancer cells and showed that it suppresses type 1 cytokine-induced expression of *CCL2, CCL5, CXCL9, CXCL10* and *IL-6* while promoting the expression of *IL-1*β. Blocking of the EGFR with cetuximab when tumor cells were stimulated with the type 1 cytokine IFNγ and TNFα resulted in the amplification of the production of the T-cell attracting chemokines and resulted in an increased migration of CD4+ and CD8+ lymphocytes in chemotaxis assays in vitro. In vivo, cetuximab treatment enhanced the serum levels of CXCL9 and CXCL10 in patients with head and neck cancer. Our data are in line with previous studies showing that EGFR mutated tumors display an uninflamed phenotype [17] and that cetuximab treatment may lead to increased T-cell infiltration in head and neck cancers [28, 29]. Whilst others observed that EGFR inhibition can lead to the direct increased secretion of proinflammatory cytokines, including IL2, IL4, IL6, IL8, GMCSF [30], our study showed an increased expression of CCL2, CCL5, CXCL9, CXCL10 after EGFR blockade only when the tumor cells were stimulated with IFNγ and TNFα. Mechanistic studies revealed that the EGFR mediated the repression of chemokine production through the activation of multiple downstream signaling pathways, predominantly via JNK and MEK1, that act simultaneously. In melanoma, the B-raf, β-catenin and PTEN-PI3K/mTOR oncogenic pathways have been identified as direct repressors of tumor-infiltration by T cells [20, 21, 31]. We show that the EGFR-signaling pathway complements this list albeit that it acts a bit different in that it suppresses the type 1 cytokine mediated amplification of tumor infiltration by T cells, via blockade of the production of T-cell attracting chemokine by tumor cells when stimulated with type 1 cytokines.

Interestingly, blocking of the downstream molecule c-RAF by GW5074 blunted the IFNγ/TNFα induced expression of the T-cell attracting chemokines while it enhanced the expression of *IL-1*β confirming earlier reports that RAF can be activated by both IFNγ [27, 32] and TNFα [33], and revealing an important role for c-RAF in relaying the

signals induced by IFNγ and TNFα that lead to cytokine production. The enhanced expression of *IL-1*β after GW5074 treatment was related to an increased phosphorylation of MEK1 and ERK1/2, indirectly confirming that activation of EGFR-downstream MEK and ERK affect IFNγ/TNFα induced chemokine secretion in head and neck cancer. Blocking of the EGFR by cetuximab enhanced the expression of the T-cell attracting chemokines but lowered the expression of *IL-1*β Potentially, this contributes to the intratumoral T cell responses as IL-1β may indirectly suppress the proliferation and function of these T cells via tumor associated fibroblasts that start to express COX-2 and PD-L1 [34]. In addition, IL-1β induces the accumulation of immune suppressive myeloid derived suppressor cells [35]. Thus, cetuximab treatment may not only amplify the attraction of T cells but also allow for the accumulation of more effective T cells in the tumor.

T cell infiltration of head and neck cancers is a positive prognostic factor in head and neck cancer [2-4, 36]. The EGFR-mediated suppression of IFNγ/TNFα mediated amplification of the T-cell infiltrate in these cancers bears strong similarities with observations in skin inflammation disorders. Also, here IFNγ and TNFα promote *de novo* synthesis of numerous chemokines responsible for the attraction of immune cells but this reaction is suppressed by active EGFR signaling. Blockade of the EGFR pathway increases chemokine production and leads to enhanced immune cell infiltration of the skin [37] Interestingly, in these skin disorders the activation of EGFR was the result of increased levels of soluble EGFR ligands produced by keratinocytes in response to IFNγ and TNFα present in the milieu [37], suggesting that EGFR-mediated suppression of chemokine production can occur because of constitutive EGFR-signaling but also as result of an adaptive negative feed-back loop. Other reported immune escape mechanisms that can play a role because of constitutive or adaptive EGFR-signaling are the expression of PD-L1 and the inhibition of antigen processing via dephosphorylation of STAT1 in cancer cells [16, 38, 39]. Notably, in one (UM-SCC47) of the

4 HNSCC cell lines studied cetuximab treatment increased the level of phosphorylated STAT1 in the nucleus.

In order to study the mechanism underlying EGFR-mediated suppression of chemokine expression we analyzed its downstream pathways. Previously, we found that EGFR was overexpressed in HPV-infected keratinocytes and resulted in the upregulation of interferon-related developmental regulator 1 (IRFD1) which suppressed IFNγ/TNFα induced chemokine expression by preventing the acetylation of RelA [40]. The involvement of IRFD1 was also studied in the HPV-negative and HPV-positive cell lines used in this study. Potentially it plays an additional role in one (UM-SCC104) of the 4 cell lines but not in the other cell lines. Furthermore, we found quite a variability in the expression and phosphorylation of the proteins downstream of the EGFR. However, in combination with the data obtained when specific pathway inhibitors were used it became clear that in all cell lines the suppression of chemokine production was mediated via the EGFR downstream molecules MEK1 and JNK. One possible acting mechanism is the destabilization of the newly synthesized mRNAs via the activation of MEK/ERK [41]. Interestingly, sustained ERK activation is also a part of the normal regulatory pathway of IFNγ signaling mediated by the suppressor of cytokine signaling 1 [32]. Another reported MEK related mechanism influencing chemokine expression is the enhanced nuclear translocation of IRF-1 [23, 42], but this was not observed by us. A third non-exclusively occurring MEK/ERK-related mechanism which may play a role is the ERK negative feedback phosphorylation that inactivates RAF signaling [43], which we have shown is important for relaying the signals of the IFNγR and TNFR in our system. Inhibition of EGFR signaling has also been reported to stimulate the expression and activation of NOX4 resulting in hydrogen peroxide-induced oxidative stress, consequently leading to activation of NFκB and AP-1 with as result the induction of pro-inflammatory cytokines. This mechanism is not likely to play a role in the suppression of IFNγ/TNFα mediated chemokine production as inhibition of AP1 or NFκB did not influence the expression levels of the tested chemokines. The

downstream molecules P38 and JNK play a crucial role in biosynthesis of cytokines [44]. Our results showed no role for P38 while JNK did influence IFNγ/TNFα mediated chemokine production. Especially in the HPV+ HNC cell lines the impact of JNK inhibition was stronger than that of MEK/ERK inhibition. JNK knock-out macrophages also display higher levels of the chemokines *CCL5, CXCL9* and *CXCL10* after stimulation with IFNγ and potentially this is related to modulation of the mRNA expression levels [45], suggesting that similar to MEK/ERK also JNK may regulate chemokine expression in the head and neck cancer cells by controlling mRNA stability.

In conclusion, we provided evidence that overexpression of EGFR in head and neck cancer cells results in the activation of multiple downstream signaling pathways that act simultaneously to repress the type 1 cytokine mediated amplification of tumor T cell infiltration by suppressing the production of T-cell attracting chemokines. Our experiments highlight a dominant role for the downstream MEK and JNK pathways in this process. Interfering with this process may increase the efficacy of current T-cell based immunotherapies, not only in head and neck cancer but also in several others types of cancer where overexpression of EGFR is found. Direct inhibition of MEK and JNK via targeted therapy is not likely to help the antitumor response as these pathways are also important for the proliferation, polarization and cytotoxic capacity of T cells [44], leaving direct blocking of the EGFR as the best possible current option.

Acknowledgements

SJAMS and MJPW were financially supported by a grant from the Dutch Cancer Society 2014-6696 to SHvdB and MJPW. WM is supported by a grant from the China Scholarship Council (201306240016).

References

1. Sankaranarayanan R, Masuyer E, Swaminathan R, Ferlay J, Whelan S. Head and neck cancer: a global perspective on epidemiology and prognosis. Anticancer research. 1998;18(6B):4779-86.

2. Nguyen N, Bellile E, Thomas D, McHugh J, Rozek L, Virani S, et al. Tumor infiltrating lymphocytes and survival in patients with head and neck squamous cell carcinoma. Head & neck. 2016;38(7):1074-84.

3. Kim HR, Ha S-J, Hong MH, Heo SJ, Koh YW, Choi EC, et al. PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. Scientific reports. 2016;6:36956.

4. Balermpas P, Michel Y, Wagenblast J, Seitz O, Weiss C, Rödel F, et al. Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer. British journal of cancer. 2014;110(2):501-9.

5. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. New England Journal of Medicine. 2010;363(1):24-35.

6. Wansom D, Light E, Worden F, Prince M, Urba S, Chepeha DB, et al. Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. Archives of Otolaryngology–Head & Neck Surgery. 2010;136(12):1267-73.

7. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay NEH, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clinical cancer research. 2006;12(2):465-72.

8. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, et al. PD-1–expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. Cancer research. 2013;73(1):128-38.

9. Welters MJ, Ma W, Santegoets SJ, Goedemans R, Ehsan I, Jordanova ES, et al. Intratumoral HPV16-specific T-cells constitute a type 1 oriented tumor microenvironment to improve survival in HPV16-driven oropharyngeal cancer. Clinical Cancer Research. 2017:clincanres. 2140.017.

10. Ferris RL, Blumenschein Jr G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. The New England journal of medicine. 2016;2016(375):1856-67.

11. Maiti GP, Mondal P, Mukherjee N, Ghosh A, Ghosh S, Dey S, et al. Overexpression of EGFR in head and neck squamous cell carcinoma is associated with inactivation of SH3GL2 and CDC25A genes. PloS one. 2013;8(5):e63440.

12. Yunhong T, Jie L, Yunming T, Guoqian Z, Xing Z, Ronghui Z, et al. Efficacy and safety of anti-EGFR agents administered concurrently with standard therapies for patients with head and neck squamous cell carcinoma: A systematic review and meta-analysis of randomized controlled trials. International journal of cancer. 2017.

13. Tejani MA, Cohen RB, Mehra R. The contribution of cetuximab in the treatment of recurrent and/or metastatic head and neck cancer. Biologics: targets & therapy. 2010;4:173.

14. Pollack BP, Sapkota B, Cartee TV. Epidermal growth factor receptor inhibition augments the expression of MHC class I and II genes. Clinical Cancer Research. 2011;17(13):4400-13.

15. Akbay EA, Koyama S, Carretero J, Altabef A, Tchaicha JH, Christensen CL, et al. Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. Cancer discovery. 2013;3(12):1355-63.

16. Concha-Benavente F, Srivastava RM, Trivedi S, Lei Y, Chandran U, Seethala RR, et al. Identification of the cell-intrinsic and-extrinsic pathways downstream of EGFR and IFNγ that induce PD-L1 expression in head and neck cancer. Cancer research. 2016;76(5):1031-43.

17. Dong Z-Y, Zhang J-T, Liu S-Y, Su J, Zhang C, Xie Z, et al. EGFR mutation correlates with uninflamed phenotype and weak immunogenicity, causing impaired response to PD-1 blockade in non-small cell lung cancer. OncoImmunology. 2017;6(11):e1356145.

18. Chong CR, Jänne PA. The quest to overcome resistance to EGFR-targeted therapies in cancer. Nature medicine. 2013;19(11):1389-400.

19. Peng W, Chen JQ, Liu C, Malu S, Creasy C, Tetzlaff MT, et al. Loss of PTEN promotes resistance to T cell–mediated immunotherapy. Cancer discovery. 2016;6(2):202-16.

20. Liu C, Peng W, Xu C, Lou Y, Zhang M, Wargo JA, et al. BRAF inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. Clinical cancer research. 2013;19(2):393-403.

21. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic [bgr]-catenin signalling prevents anti-tumour immunity. Nature. 2015;523(7559):231-5.

22. Tummers B, Goedemans R, Pelascini LP, Jordanova ES, Van Esch EM, Meyers C, et al. The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NFκB activation. Nature communications. 2015;6:6537.

23. Zaheer RS, Koetzler R, Holden NS, Wiehler S, Proud D. Selective transcriptional down-regulation of human rhinovirus-induced production of CXCL10 from airway epithelial cells via the MEK1 pathway. The Journal of Immunology. 2009;182(8):4854-64.

24. Carrigan SO, Junkins R, Yang YJ, MacNeil A, Richardson C, Johnston B, et al. IFN regulatory factor 3 contributes to the host response during Pseudomonas aeruginosa lung infection in mice. The Journal of Immunology. 2010;185(6):3602-9.

25. Wagner EF, Nebreda ÁR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nature Reviews Cancer. 2009;9(8):537-49.

26. Belka C, Wiegmann K, Adam D, Holland R, Neuloh M, Herrmann F, et al. Tumor necrosis factor (TNF)-alpha activates c-raf-1 kinase via the p55 TNF receptor engaging neutral sphingomyelinase. The EMBO journal. 1995;14(6):1156.

27. Zibara K, Zeidan A, Bjeije H, Kassem N, Badran B, El-Zein N. ROS mediates interferon gamma induced phosphorylation of Src, through the Raf/ERK pathway, in MCF-7 human breast cancer cell line. Journal of cell communication and signaling. 2017;11(1):57-67.

28. Jie H-B, Schuler PJ, Lee SC, Srivastava RM, Argiris A, Ferrone S, et al. CTLA-4+ regulatory T cells increased in cetuximab-treated head and neck cancer patients suppress NK cell cytotoxicity and correlate with poor prognosis. Cancer research. 2015;75(11):2200-10.

29. Trivedi S, Srivastava RM, Concha-Benavente F, Ferrone S, Garcia-Bates TM, Li J, et al. Anti-EGFR targeted monoclonal antibody isotype influences antitumor cellular immunity in head and neck cancer patients. Clinical Cancer Research. 2016;22(21):5229-37.

30. Fletcher EV, Love-Homan L, Sobhakumari A, Feddersen CR, Koch AT, Goel A, et al. EGFR inhibition induces proinflammatory cytokines via NOX4 in HNSCC. Molecular Cancer Research. 2013;11(12):1574-84.

31. Frederick DT, Piris A, Cogdill AP, Cooper ZA, Lezcano C, Ferrone CR, et al. BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. Clinical cancer research. 2013;19(5):1225-31.

32. Madonna S, Scarponi C, De Pità O, Albanesi C. Suppressor of cytokine signaling 1 inhibits IFN-γ inflammatory signaling in human keratinocytes by sustaining ERK1/2 activation. The FASEB Journal. 2008;22(9):3287-97.

33. Modur V, Zimmerman GA, Prescott SM, McIntyre TM. Endothelial Cell Inflammatory Responses to Tumor Necrosis Factor α Ceramide-dependent and-independent Mitogen-activated Protein Kinase Cascades. Journal of Biological Chemistry. 1996;271(22):13094-102.

34. Khalili JS, Liu S, Rodríguez-Cruz TG, Whittington M, Wardell S, Liu C, et al. Oncogenic BRAF (V600E) promotes stromal cell-mediated immunosuppression via induction of interleukin-1 in melanoma. Clinical Cancer Research. 2012;18(19):5329-40.

35. Elkabets M, Ribeiro VS, Dinarello CA, Ostrand-Rosenberg S, Di Santo JP, Apte RN, et al. IL-1β regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function. European journal of immunology. 2010;40(12):3347-57.

36. de Ruiter EJ, Ooft ML, Devriese LA, Willems SM. The prognostic role of tumor infiltrating T-lymphocytes in squamous cell carcinoma of the head and neck: A systematic review and meta-analysis. Oncoimmunology. 2017;6(11):e1356148.

37. Mascia F, Mariani V, Girolomoni G, Pastore S. Blockade of the EGF receptor induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation. The American journal of pathology. 2003;163(1):303-12.

38. Srivastava RM, Trivedi S, Concha-Benavente F, Hyun-Bae J, Wang L, Seethala RR, et al. STAT1-induced HLA class I upregulation enhances immunogenicity and clinical response to anti-EGFR mAb cetuximab therapy in HNC patients. Cancer immunology research. 2015;3(8):936-45.

39. Concha-Benavente F, Srivastava RM, Ferrone S, Ferris RL. EGFR-mediated tumor immunoescape: the imbalance between phosphorylated STAT1 and phosphorylated STAT3. Oncoimmunology. 2013;2(12):e27215.

40. Tummers B, Goedemans R, Pelascini LP, Jordanova ES, Van Esch EM, Meyers C, et al. The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NFκB activation. Nature communications. 2015;6.

41. Pastore S, Mascia F, Mariotti F, Dattilo C, Mariani V, Girolomoni G. ERK1/2 regulates epidermal chemokine expression and skin inflammation. The Journal of Immunology. 2005;174(8):5047-56.

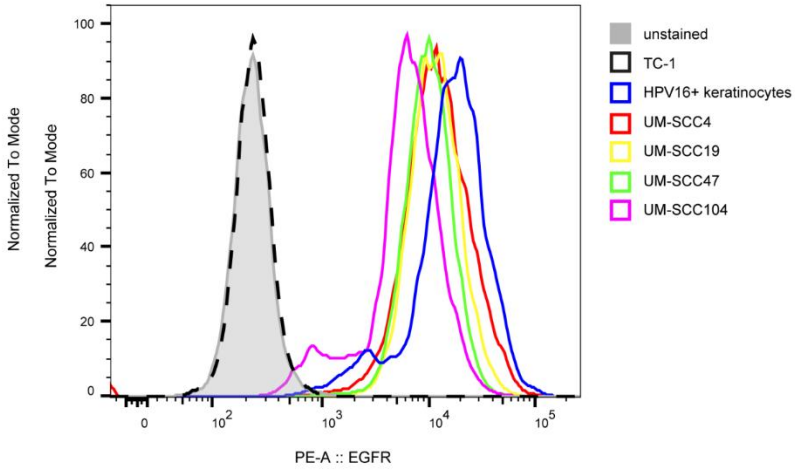
42. Kalinowski A, Ueki I, Min-Oo G, Ballon-Landa E, Knoff D, Galen B, et al. EGFR activation suppresses respiratory virus-induced IRF1-dependent CXCL10 production. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2014;307(2):L186-L96.

43. Dhillon AS, von Kriegsheim A, Grindlay J, Kolch W. Phosphatase and feedback regulation of Raf-1 signaling. Cell Cycle. 2007;6(1):3-7.

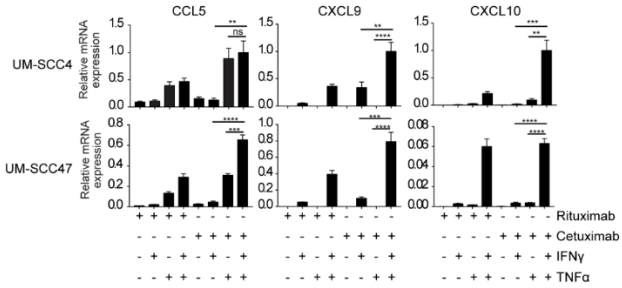
44. Liu Y, Shepherd EG, Nelin LD. MAPK phosphatases-regulating the immune response. Nature Reviews Immunology. 2007;7(3):202-13.

45. Valledor AF, Sánchez-Tilló E, Arpa L, Park JM, Caelles C, Lloberas J, et al. Selective roles of MAPKs during the macrophage response to IFN-γ. The Journal of Immunology. 2008;180(7):4523-9.

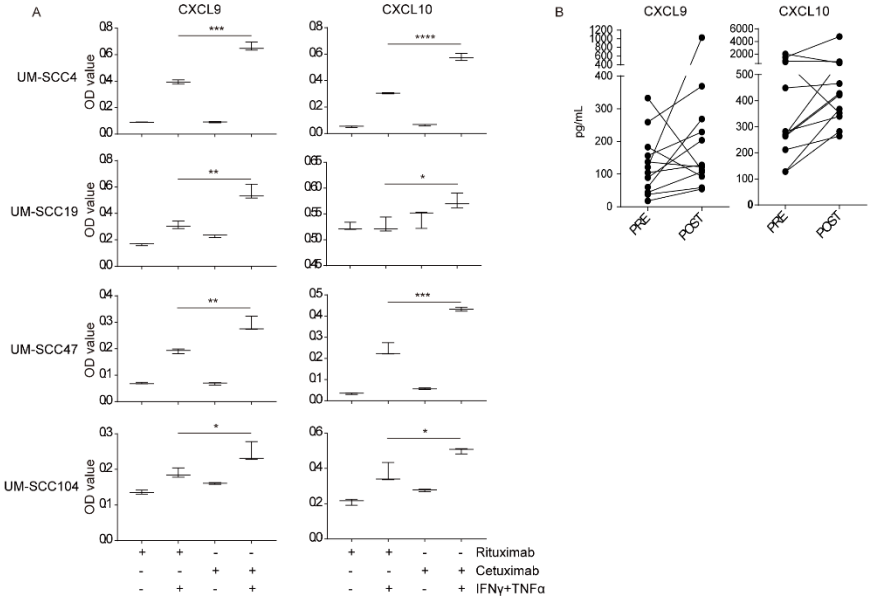
SUPPLEMENTARY INFORMATION



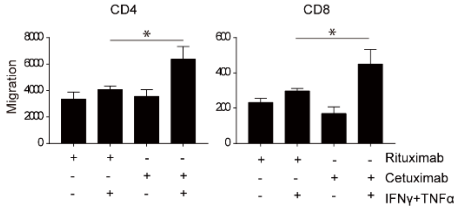
Supplementary Figure 1 Histogram of EGFR surface protein expression on two HPV- HNC cell lines UM-SCC4 and UM-SCC9 and two HPV+ HNC cell line UM-SCC47 and UM-SCC104 as determined by flow cytometry. TC-1 is the mouse cell line as negative control which can not be stained by the antibody, HPV16+ keratinocytes as the positive control.



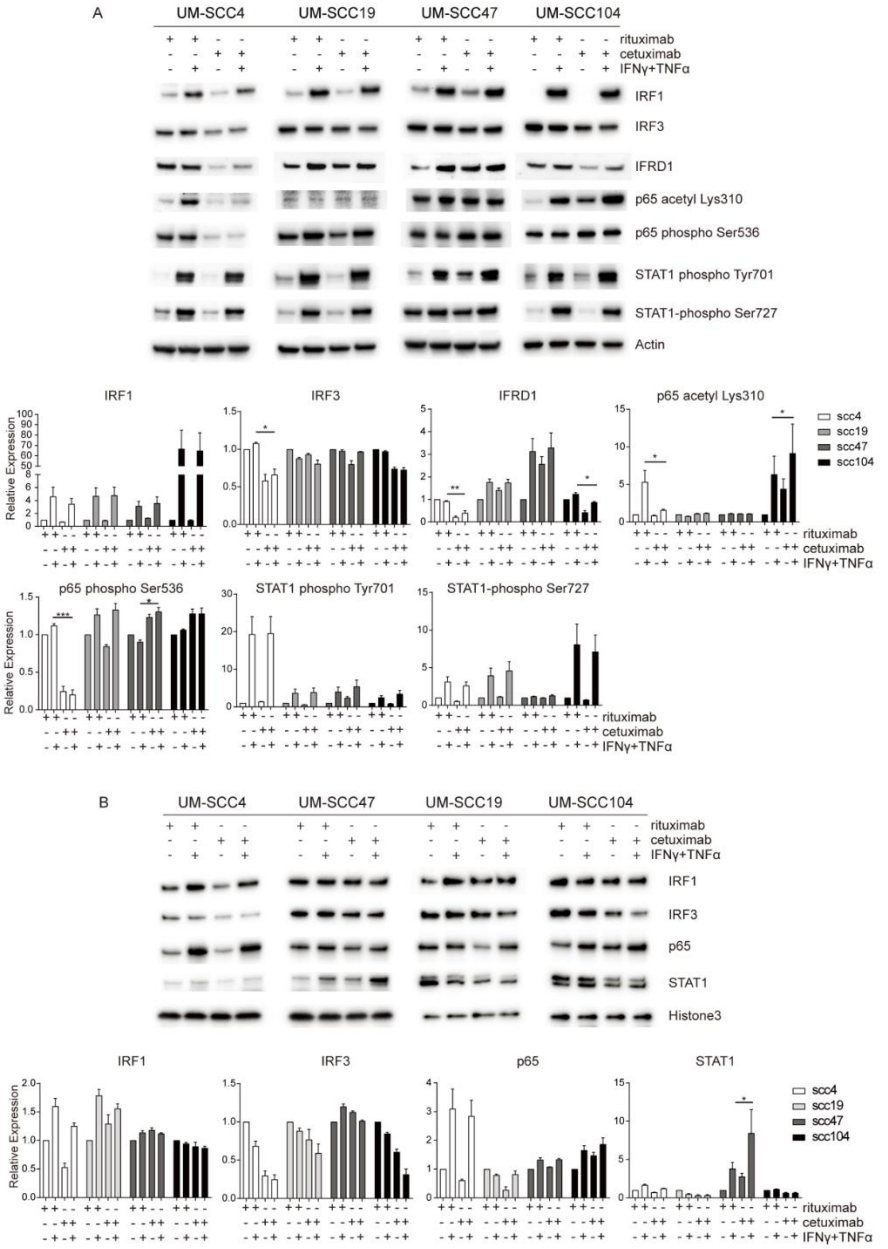
Supplementary Figure 2 HPV- HNC cell line UM-SCC4 and HPV+ HNC cell line UM-SCC47 were stimulated with 1µg/mL rituximab or 1µg/mL cetuximab as indicated. After 48h, the cells were treated with 50IU/mL IFNγ and/or 30ng/ml TNFα as indicated for 24h, the expression levels of genes as indicated were determined by RT-qPCR. Gene expression was normalized against GAPDH mRNA levels. Similar results were observed in two independent experiments.P value were determined by unpaired t-tests. Ns: no signifcance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.



Supplementary Figure 3 (A)Two HPV- HNC cell lines (UM-SCC4 and UM-SCC19) and two HPV+ HNC cell line (UM-SCC47 and UM-SCC104) were stimulated with 1µg/mL rituximab or 1µg/mL cetuximab as indicated. After 24h, the cells were treated with 50IU/mL IFNγ and 30ng/ml TNF α as indicated for 48h. The OD value in supernatants of CXCL9 and CXCL10 was determined by Enzyme-linked immunosorbent assay. P value were determined by unpaired t-tests. Ns: no signifcance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (B)Peripheral venous blood samples were obtained from HNC patients with stage III/IVA disease, receiving neoadjuvant single-agent cetuximab in a prospective phase II clinical trial. A representative pre- and post-treatment sample from 12 randomly selected patients (all Caucasian, age 49-93 years old) were used for cytokine determination.

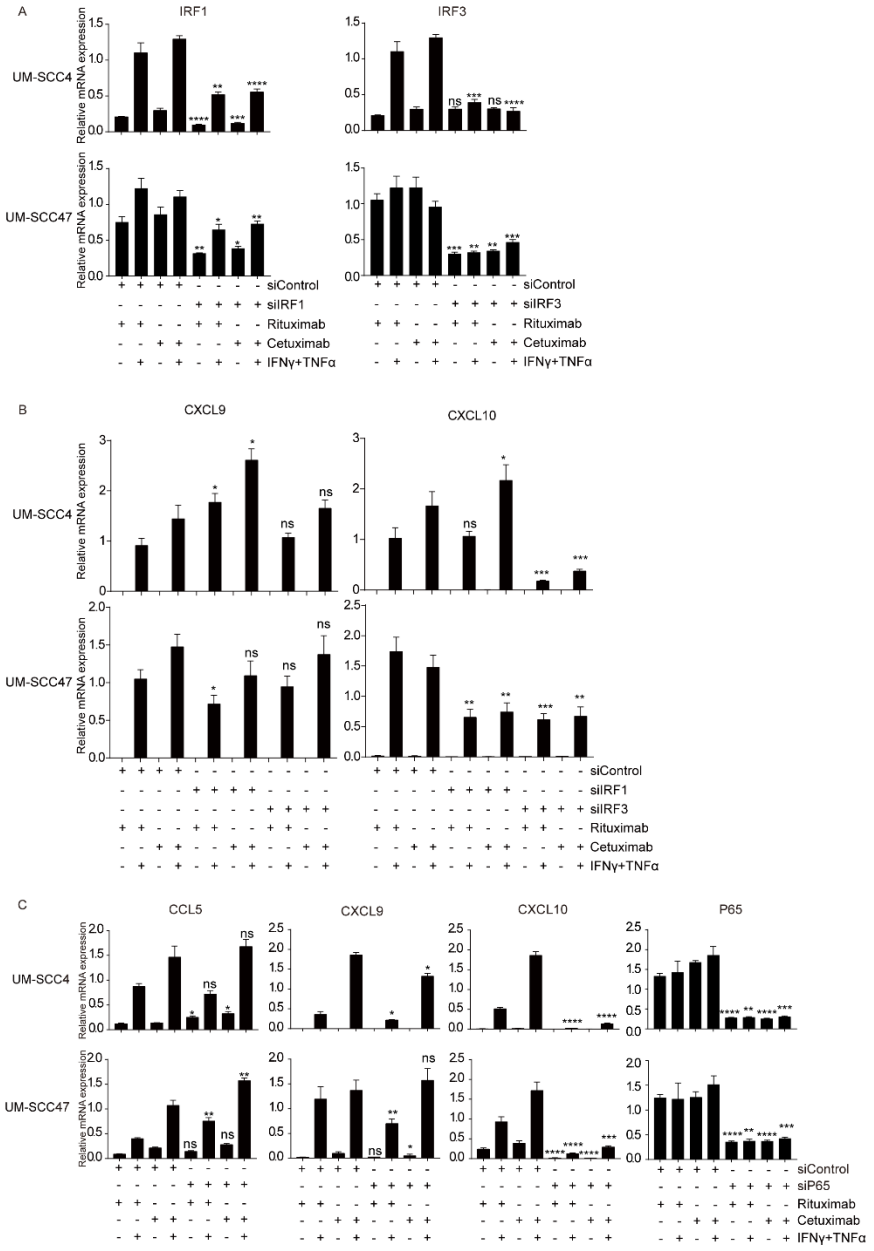


Supplementary Figure 4 UM-SCC4 was stimulated with 1µg/mL rituximab or 1µg/mL cetuximab as indicated. After 24h, the cells were treated with 50IU/mL IFNγ and 30ng/ml TNFα as indicated for 48h. CD14-depleted PBMCs migration towards supernatants was determined by transwell assay. Number of CD4+ and CD8+ T cells within migrated CD14-depleted PBMC was determinded by flow cytometry. P value were determined by unpaired t-tests. Ns: no signifcance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.



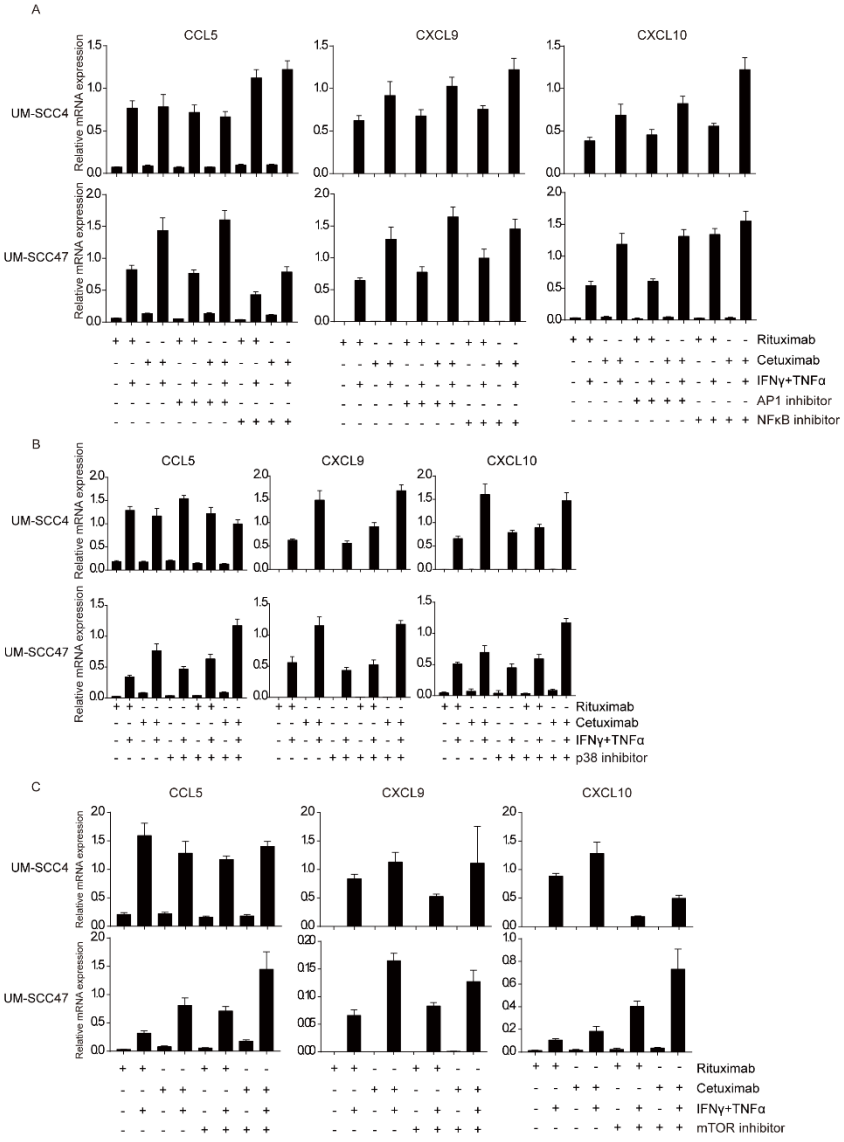
Supplemental Figure 5 (A) Two HPV- HNC cell lines (UM-SCC4 and UM-SCC19) and two HPV+ HNC cell line (UM-SCC47 and UM-SCC104) were stimulated with 1μg/mL rituximab or 1μg/mL cetuximab as indicated. After 48h, the cells were treated with 50IU/mL IFNγ and 30ng/mL TNFα as indicated for 24h. The protein expression levels of IRF1, IRF3, IFRD1, p65-acetylation, p65-phosphorylation, phosphor-STAT1 Tyr701 and Phospho-STAT1 Ser727 as detected by Western blotting(WB) in whole cell extracts. β-actin served as loading control.

(B)Two HPV- HNC cell lines (UM-SCC4 and UM-SCC19) and two HPV+ HNC cell line (UM-SCC47 and UM-SCC104) were stimulated with 1ug/mL rituximab or 1ug/mL cetuximab as indicated. After 48h, the cells were treated with 50IU/mL IFNγ, 30ng/ml TNFα as indicated for 24h. The protein expression levels of IRF1, IRF3, p65, STAT1 as detected by Western blotting (WB) in nuclear extracts. Histone3 served as loading control.

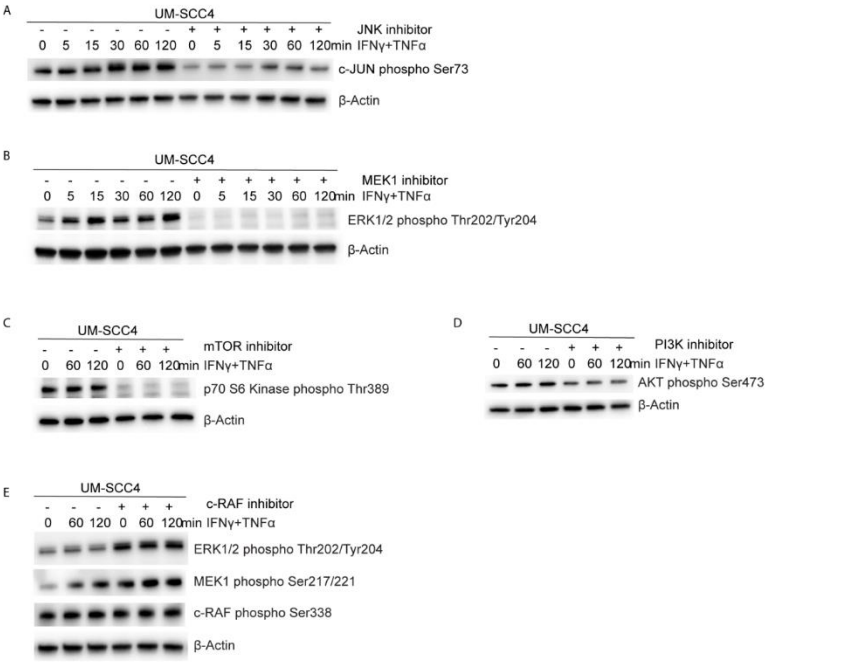


Supplementary Figure 6 (A,B)Expression of CXCL9 and CXCL10 in HPV- HNC cell line UM-SCC4 and HPV+ HNC cell line UM-SCC47 transfected with control siRNA (siControl) or siRNA targeting IRF1 or IRF3 stimulated with or without 1µg/mL rituximab or 1µg/mL cetuximab as indicated. After 24h, the cells were treated with 50IU/mL IFNγ and 30ng/ml TNFα as indicated for 24h. Gene expression was normalized against GAPDH mRNA levels and standardized against siControl. Similar results were observed in two independent experiments. P value were determined by unpaired t-tests of siControl group compared with siIRF1 and siIRF3 group respectivley. Ns: no signifcance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

(C)Expression of CCL5, CXCL9 and CXCL10 in HPV- HNC cell line UM-SCC4 and HPV+ HNC cell line UM-SCC47 transfected with control siRNA (siControl) or siRNA targeting P65 stimulated with or without 1µg/mL rituximab or 1µg/mL cetuximab as indicated. After 24h, the cells were treated with 50IU/mL IFNγ and 30ng/ml TNFα as indicated for 24h. Gene expression was normalized against GAPDH mRNA levels and standardized against siControl. Similar results were observed in two independent experiments. P value were determined by unpaired t-tests of siControl group compared with siIRF1 and siIRF3 group respectivley. Ns: no signifcance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.



Supplementary Figure 7 HPV- HNC cell line UM-SCC4 and HPV+ HNC cell line UM-SCC47 were stimulated with 1μg/mL rituximab or 1μg/mL cetuximab as indicated for 72h, (A)10μM JSH-23(NFkB inhibitor) and 20μM T-5224(AP-1 inhibitor) (B)0,5μM pamapimod(P38 inhibitor) (C)50nM Rapamycin(mTOR inhibitor) as indicated for 48h, 50IU/mL IFNγ and 30ng/ml TNFα as indicated for 24h. The expression levels of CCL5, CXCL9 and CXCL10 were determined by RT-qPCR. Gene expression was normalized against GAPDH mRNA levels. Similar results were observed in two independent experiments.



Supplementary Figure 8 UM-SCC4 was stimulated with (A)20μM SP600125 (JNK inhibitor), (B)50μM PD98059 (MEK1 inhibitor), (C)50nM Rapamycin (mTOR inhibitor), (D)25μM LY-294,002 (PI3K inhibitor), (E)20μM GW5074 (c-RAF inhibitor) for 2h, then the cells were treated with 50IU/mL IFNγ and 30ng/ml TNFα for different time points as indicated. The expression levels of proteins as indicated were detected by Western blotting (WB) in whole cell extracts. β-actin served as loading control. Similar results were observed in two independent experiments.

Chapter 6

Discussion

Discussion

Human papillomavirus is one of the most common sexually transmitted pathogens in the world [1]. A persistent HPV infection can lead to the development of malignancies. The immune system plays a crucial role in controlling the progression of the disease and about 90% of the infections are cleared within three years, while 10% persist and less than 1% develop into cervical cancer [2]. A type 1 T-cell response is important for the control of HPV infections and individuals with a suppressed T-cell response display more infections [3, 4]. Furthermore, an HPV-specific Th1 immune response is frequently detected in healthy donors [5, 6] and the induction of strong HPV-specific type 1 T-cell responses by therapeutic vaccination is associated with regression of CIN or VIN lesions [7-12]. Finally, also in cancer, a type 1 immune contexture is associated with a better response to standard therapy and immunotherapy [13], including CxCa and OPSCC [13, 14]. In this thesis, we study the mechanisms allowing HPV-infected and -transformed cells to resist the attack of a type 1 T-cell response.

1. Human papillomavirus-infected cells are less sensitive to the antiproliferative effects of IFNγ

Human papillomavirus has developed multiple direct and indirect mechanisms to influence cell proliferation. The HPV E6 and E7 oncoproteins act to increase the proliferation of HPV-infected cells in the epithelium. Human papillomavirus interferes with the normal terminal differentiation process, thereby increasing the number of HPV-infected cells, which eventually produce more infectious virions [15-17]. Under certain conditions, the E2 protein can induce growth arrest, cell senescence and apoptotic cell death [18-20]. The receptors for IFNγ and TNFα are widely distributed among all nucleated cells, and activation of these receptors may have antiproliferative and proapoptotic effects. Regarding apoptosis, IFNγ induces IRF1 which reduces BCL2 and BAK, leading to the release of cytochrome C from the mitochondria and caspases, resulting in apoptosis [21]. Furthermore, IFNγ may trigger tumor cells to produce high concentrations of RNI and ROS, with apoptosis of the cell as a

result [22]. IFNγ is also reported to induce autophagy in HCC [23] and has an inhibitory effect on proliferation. Binding of IFNγ to the IFNγ receptor (IFNγR) leads to JAK1/2-mediated STAT1 phosphorylation, dimerization and nuclear translocation, which results in interferon-stimulated gene (ISG) expression [24]. IFNγ has been shown to induce growth arrest and differentiation of KCs [25, 26], as well as the arrest of cancer growth by IFNγ downregulating cyclins E and A, thereby inhibiting tumor growth [27]. Furthermore, activated STAT1 interacts with cyclins D1/CDK4, resulting in cell-cycle arrest [28]. Moreover, IFNγ has been shown to upregulate the cell-cycle inhibitors p27 and p21, which suppress the activity of E2F transcription factor and inhibit the activation of genes involved in cell proliferation [29]. A previous study shows that STAT1 is selectively suppressed by HPV to allow for HPV genome amplification and maintenance of episomes [30]. In chapter 2, we confirm that HPV downregulated STAT1 expression but also show that the inhibition of STAT1 was not complete, as IFNγ was still able to induce phosphorylation of STAT1. Importantly, we found that HPV resisted the antiproliferative effects of IFNγ through downregulation of the STAT1 downstream targets *IFITM1* and *RARRES1* (chapter 2). IFNγ-mediated activation of IFITM1 results in the inhibition of ERK phosphorylation, thereby suppressing MAPK signaling. IFITM1 also increases the stability of p53 and arrests the cell cycle at G1 phase [31]. Indeed, in our experiments, IFNγ treatment reduced about 50% of the cells in the S phase in normal KCs, though this was not observed in the HPV-positive KCs, indicating that HPV resisted the anti-proliferation effects of IFNγ by downregulating the expression of *IFITM1*. Furthermore, we found that the expression of *RARRES1* was significantly decreased in the HPV+ KCs. *RARRES1* is considered as a putative tumor-suppressor gene, largely based on the hyper-methylation of its promoter in many tumor types and ageing normal tissues [32-35]. Expression of RARRES1 inhibits cell growth in prostate and endometrial cancer cells [36, 37]. Moreover, we found that HPV significantly increased the expression of the proliferating cell nuclear antigen (PCNA), which is essential for the DNA replication of small DNA tumor viruses associated with HPV infection and the

progression of cervical intraepithelial neoplasia (CIN) [38], and is considered to be a marker for cell proliferation in various cancers [39].

Thus, under normal physiological conditions, Th1 cells may migrate to HPV-infected lesions and secrete IFNγ to control viral replication by inhibiting cell proliferation of HPV-infected cells via the increased expression of *IFITM1* and *RARRES1*. According to our results, HPV may escape these effects of immune surveillance by downregulating the expression of these anti-proliferation genes and upregulating the proliferation marker *PCNA*. As *RARRES1* and *PCNA* also play a role in oncogenesis, the alteration of their expression by HPV may also contribute to the malignant transformation of the infected KCs. Hence, if the cytokines produced by type 1 T cells cannot interfere sufficiently with cell growth to prevent virus production or division of transformed cells, there is a need to kill the infected or transformed cells by induction of cell death.

2. Human papillomavirus impairs TNFα/IFNγ-induced necroptosis

Necrosis is an inflammatory type of cell death characterized by cell swelling, loss of plasma membrane integrity and release of cytosolic contents into the extracellular space [40], and plays a role as a host defense strategy to prevent viral infections [41]. The murine cytomegalovirus [42, 43] and influenza A virus (IAV) [44-46] activate DAI-dependent necroptosis via RIPK3. Reovirus induces caspase-independent cell death [47], which forms part of the mechanism that leads to immune control of these viral infections. In an attempt to prevent the attraction of the immune system, many viruses have developed mechanisms to suppress necroptosis. Herpes simplex virus 1 (HSV-1) ICP6 and herpes simplex virus 2 (HSV-2) ICP10 proteins prevent necroptosis in human cells by inhibiting the interaction between the receptor-interacting protein kinases RIP1 and RIPK3 [48]. Human cytomegalovirus suppresses RIPK3-dependent necroptosis [49].

In chapter 2, we examine IFNγ+TNFα-mediated apoptosis in both KCs and HPV+ KCs. Our data suggest that IFNγ+TNFα alone did not cause substantial apoptotic cell death in either of these cells. Necroptosis can be induced by IFNγ and TNFα when cIAPs and caspase-8 are

inhibited by BV6 and zVAD-fmk, respectively. We examined the expression of cIAPs and caspase-8 in the normal KCs and HPV+ KCs, and found that both of these molecules were still present in HPV+ KCs. In order to prime KCs and HPV+ KCs for necroptosis, the cells were treated with BV6 and zVAD-fmk. However, IFNγ+TNFα-induced necroptosis was significantly higher in KCs than in HPV+ KCs. We show that downregulation of RIPK3, which is the key component of the necrosome, was the underlying mechanism (chapter 2). As necroptosis is key in initiating the adaptive immune response for the control of viral infections, HPV evolved to remain stealthy and evade necroptosis induced by the Th1 cytokines IFNγ and TNFα. Moreover, Fas, granzymes and perforins are important mediators of cell death used by type 1 T cells. Others found that RIPK3 knockout endothelial cells resisted necroptosis induced via these molecules [50]. As RIPK3 is downregulated by HPV, HPV+ KCs may also partly resist the cytotoxicity effects of T cells' Fas, granzymes and perforins.

We found that RIPK3 was downregulated at the transcription level by HPV, indicating that methylation may be involved. We found that treatment of the cells with DZNep, which is a global inhibitor of histone methyltransferases that depletes EZH2, restored the expression of RIPK3 in HPV+ KCs. As a result, DZNep also restored the sensitivity of IFNγ- and TNFα-induced necroptosis in HPV+ KCs. However, catalytic EZH2-inhibitor GSK503 did not restore RIPK3 expression, indicating that EZH2 indirectly suppresses RIKP3 expression or that other histone methyltransferases are also involved. We tested about 40 methyltransferases in the KCs and HPV+ KCs, and found that HPV altered about eight methyltransferases in KCs. Therefore, the downregulation of RIKP3 by HPV may be a complex effect due to HPV's alteration of several methyltransferases (chapter 2).

Unlike apoptosis, necroptosis is a highly inflammatory process. It mediates the release of intracellular DAMPs, including interleukin 1a, HMGB1, uric acid, ATP and DNA, resulting in the recruitment of proinflammatory cell types to sites of infection [51]. In this process, RIPK3 also drives the production of IL-1β [52], which is an important

factor for the initiation of inflammation and the activation of immune cells such as macrophages and T cells [53]. In HPV16-immortalized human KCs, IL-1β secretion is impaired because the pro-IL-1β is degraded in a proteasome-dependent manner, mediated via the ubiquitin ligase E6-AP and p53 [54]. Biopsies from different progression states (cervical intraepithelial neoplasia, CIN I-III) and cervical cancer show a decrease of pro-IL-1β protein expression with an increased progression stage [54]. Thus, HPV prevents IFNγ and TNFα-mediated necroptosis, which may be one of the mechanisms contributing to the escape of HPV from immune surveillance and could explain why HPV behaves as a stealthy virus.

3. HPV-positive head and neck cancer is not sensitive to IFNγ- and TNFα-induced necroptosis, is there a need for chemotherapy co-treatment?

Subsequently seeking to understand whether a similar mechanism plays a role in cancer and whether this is HPV specific, we studied oropharyngeal cancers, as half of them are induced by HPV. Moreover, HPV-positive OPSCC displays a far better prognosis than HPV-negative tumors after (chemo)radiation therapy [55, 56], which is associated with a strong adaptive immune response at the tumor site [56-58]. In chapter 4, we show that the majority of HPV-positive OPSCCs is infiltrated with HPV16-specific T cells, producing high concentrations of IFNγ, TNFα and IL17A. By contrast, the tumor-infiltrating T cells from the group of patients who lacked an HPV-specific immune response displayed a low production of IFNγ and IL17A, while the production of IL-5 was increased, suggesting a shift towards a type 2 cytokine profile. The presence of HPV16-specific Th1/Th17 cells was strongly associated with better survival, suggesting that a Th1/Th17 immune response mediated the control of cancer cells.

To understand whether Th1/Th17 cells may contribute to necroptosis, we examined several proteins related to the apoptosis and necroptosis in the TNFR pathway in HPV- and HPV+ OPSCC cell lines in vitro. The different cell lines displayed some variance in the expression of these proteins. TRAF2 is considered as an antiapoptosis

protein that recruits cIAP1/2 to promote NF-κB signaling [59]. TRAF2 is an NF-κB-activating oncogene in epithelial cancers which is amplified and rearranged in 15% of human epithelial cancers [60]. TRAF2 was downregulated by IFNγ and TNFα treatment in UM-SCC19 (HPV-), UM-SCC47 (HPV+) and UM-SCC104 (HPV+), while TRAF2 expression was already relatively low in two other HPV cell lines (UM-SCC6 and UM-SCC4). cIAP1 and cIAP2 (cIAP1/2) are cellular inhibitors of apoptosis proteins, the amplification or genetic mutation of which has been associated with cancers and may promote tumor cell survival [61]. cIAP1/2 expression was at a low level in UM-SCC4 and UM-SCC104. Fas-associated death domain protein (FADD) is a classical adaptor protein mediating apoptotic stimuli-induced cell death. In cancer, however, FADD protects pancreatic cancer cells from drug-induced apoptosis [62]. Fas-associated death domain protein also plays a role in necroptosis. When caspase-8 is inhibited by inhibitors or the depletion of FADD by shRNA, cells undergo necroptosis via RIP1-RIPK3-complex formation and the activation of downstream pathways [63-66]. We found that FADD expression in UM-SCC6, UM-SCC47 and UM-SCC104 was relatively low and that RIPK3 is absent in the HPV-positive HNC cell lines. Furthermore, we examined whether treatment with the Th1 cytokines IFNγ and TNFα induced apoptosis and necroptosis in these cell lines. Similar to our results with KCs, no significant apoptosis was induced and the HPV-positive head and neck cancer cells were less sensitive to IFNγ- and TNFα-induced necroptosis. RIPK3 expression is absent in various cancer cell lines, including the HPV-positive cell line Hela, due to the genomic methylation at the site of the RIPK3. The loss of RIPK3 expression in many cancer cell lines is due to hypermethylation in the promoter region, which was high for RIPK3 [67]. The hypomethylating agent 5-AAD can restore the expression of RIPK3 and consequently increase the sensitivity to chemotherapeutics in a RIPK3-dependent manner [68]. It needs to be tested whether the absence of RIPK3 in the HPV-positive HNC cell line is due to DNA methylation or, similar to HPV-positive KCs, is the result of histone methylation.

Many types of cancer cell lines can undergo necroptosis by classic necroptosis inducers and existing chemotherapeutic agents, including colorectal cancer, leukemia, multiple myeloma, lung cancer, ovarian cancer, breast cancer, hepatocellular carcinoma, bladder carcinoma, head and neck carcinoma, glioblastoma, cervical cancer and neuroblastoma [69]. Necroptosis is activated in response to many chemotherapeutic agents and contributes to chemotherapy-induced cell death [68]. Among the abovementioned cancer cells, colorectal cancer cells and hematopoietic neoplasms (e.g., leukemia and multiple myeloma) appear to be more sensitive and responsive to necroptosis inducers [69]. Thus, triggering necroptosis may be an alternative way to eradicate apoptosis-resistant cancer cells. However, numerous cancer cell lines develop mechanisms to evade necroptosis. Similar to the HPV-positive head and neck cancer cell lines UM-SCC47 and UM-SCC104, the cervical cancer cell line Hela, which is also HPV positive, is resistant to necroptosis due to the low level of RIPK3. In primary colon cancer tissues, RIPK1 and RIPK3 are downregulated [70], similarly to the OPSCC cell line UM-SCC6. Stimulation of cervical cancer cells can occur with poly I:C-induced necroptotic cell death but relies on the expression of RIPK3 [71], which is known to gradually decrease during cervical carcinogenesis [54]. In acute myeloid leukemia samples, RIPK3 is decreased without a significant decrease of RIPK1 [72]. In addition, RIPK3 and CYLD are markedly downregulated in chronic lymphocytic leukemia, which is resistant to TNFα and zVAD-induced necroptosis [73]. RIPK3-mediated phosphorylation of mixed lineage kinase domain-like (MLKL) protein triggers necroptosis and leads to plasma-membrane disruption [74]. Reduced MLKL is found in pancreatic adenocarcinoma and is associated with decreased overall survival [75]. However, MLKL expression was high and not altered in our OPSCC cell lines (chapter 4). Resistance to cell death is one of the hallmarks of a cancer cell and tumor formation often selects against the expression of cell death proteins [76]. While RIPK3 expression is lost in HPV-positive OPSCC cell lines and many other cancer cell lines [68], RIPK3 is present in normal tissue and primary cells [68], which suggests that RIPK3

expression is negatively selected during initial tumor development or growth.

To improve necroptotic cell death, several drugs can be used. Breast cancer cells MCF-7 overexpress Bcl-2 and are resistant to proapoptosis drugs. Shikonin, a naturally occurring naphthoquinone, induces necroptotic cell death in MCF-7 [77]. Obatoclax, a putative antagonist of Bcl-2 family members, triggers autophagy-dependent necroptosis to reverse glucocorticoids resistance in acute lymphoblastic leukemia [78]. IAP antagonist with caspase-inhibitor zVAD treatment induces TNF-dependent necroptotic death in cisplatin and IAP-antagonist-resistant ovarian carcinoma cell lines [79]. However, the caspase inhibitors also inhibit T-cell proliferation, thus making it inadvisable to combine zVAD with immunotherapy [80, 81]. RIPK3 expression may be restored in most cells by the use of simple hypomethylating agents such as 5-AD, which is far more effective when combined with other chemotherapeutic drugs [68]. Hence, the RIPK3 expression status in cancer cells may critically influence the outcome of immunotherapeutic approaches and should therefore be assessed prior to immunotherapy.

To test the potential effect of the OPSCC-infiltrating Th1/Th17 cell-produced cytokines IFNγ and TNFα on tumor cell proliferation, we used the supernatant from antigen-stimulated HPV-specific Th1 or Th17 cells. This revealed a reduction in cell proliferation and an increase in the expression of the antiproliferative genes *IFITM1* and *RARRES1*, both of which have antiproliferative effects, suggesting that these cytokines hamper the proliferation not only of HPV-infected KCs but also of OPSCC (chapter 4). However, there was quite some variability between the different OPSCC cell lines that were tested. UM-SCC4 (HPV-), UM-SCC19 (HPV-) and UM-SCC47 (HPV+) were more sensitive to cytokine treatment compared to UM-SCC6 (HPV-) and UM-SCC104 (HPV+). It is probable that multiple mechanisms regulate the proliferation of and contribute to cell death induced by IFNγ and TNFα are involved. We found that IRF1 expression, which may lead to apoptosis [21], was significantly upregulated by IFNγ and TNFα in our OPSCC cell lines. STAT1 expression, which via the cyclins D1/CDK4

may arrest the cell cycle [28], was also significantly increased by IFNγ and TNFα in all OPSCC cancer cell lines. TNFα has multiple effects on the cancer cells. We found that TNFα alone did not cause a significant increase of apoptosis in OPSCC cell lines, but experiments in mice indicate that together with cisplatin it could synergize to induce apoptosis [82]. Cisplatin is the chemotherapeutic drug for the treatment of OPSCC. The combination of TNFα and cisplatin resulted in an increased percentage of apoptotic tumor cells and especially in the HPV-positive cell lines, as no synergistic effect was observed the HPV-negative cell lines, probably because cisplatin alone efficiently caused cell death in most HPV-negative cells. TNFα was also shown to enhance the anti-cancer effects of doxorubicin through suppressing the antiapoptotic activity of p21- and p53-deficient cancers [83].

4. The EGFR pathway suppresses the amplification of T-cell infiltration.

We show that head and neck cancers could resist the attack of type 1 T cells by interfering with mechanisms of cell proliferation and cell death. From our studies in KC, we obtained evidence supporting that the EGFR pathway is upregulated by HPV and interferes with the IFNγ and TNFα-induced expression of cytokines and chemokines, which may attract T cells [84]. The EGFR is frequently overexpressed in the cancers of patients with poor prognosis and is found to be overexpressed in 80–90% of HNSCC [85]. Therefore, as discussed in chapter 5, we examined the phosphorylation of proteins downstream of EGFR after treatment with cetuximab and found that cetuximab blocked most of the downstream pathways of EGFR, including the RAF-MEK-ERK, AKT-mTOR and MAPK pathways. We also found that inhibition of EGFR by cetuximab combined with IFNγ and TNFα led to increased cytokine production, including CCL5, CXCL9 and CXCL10, which function as T-cell-attracting chemokines to tumor sites. Chemotaxis assays in vitro confirmed that more lymphocytes migrated after the treatment of tumor cells with cetuximab and IFNγ and TNFα. This is coherent with the observation that the presence of an activating EGFR mutation is related to a lower T-cell infiltration of

human tumors [86]. Moreover, previous studies reveal that EGFR has important immune-regulatory effects. Activation of EGFR repressed the expression of MHC class I and II [87]. Overexpressed EGFR significantly correlated with JAK2 and PD-L1 expression in a large cohort of HNC specimens and PD-L1 expression was induced in an EGFR- and JAK2/STAT1-dependent manner [88]. In lung tumors, the expression of mutant EGFR in bronchial epithelial cells induced the expression of PD-L1, which was reduced by EGFR inhibitors in non-small cell lung cancer cell lines. Furthermore, the blockade of PD1 improved survival of mice in EGFR-driven murine lung tumors [89]. Together, these data suggest that EGFR has negative effects on the recruitment and effector function of T-cell immunity. Although not formerly proven in humans, data in mice suggest that the clinical effect of effective EGFR blockade indeed depends on T-cell immunity. Depletion of either $CD8^+$ or $CD4^+$ T cells was reported to abrogate the beneficial effects of EGFR inhibitor treatment in mice [90].

Importantly, we found that cetuximab alone did not significantly alter chemokine expression. Only when combined with the Th1 cytokines IFNγ and TNFα did EGFR blocking by cetuximab increase cytokine expression (chapter 5). As patients whose OPSCCs are infiltrated with type 1 T cells display far better survival, the presence of a type 1 T-cell response may improve the anti-tumor effects of EGFR inhibition. Indeed, TNFα was shown to enhance the tumor-regression effects of monoclonal antibodies against EGFR to cancer cell xenotransplants, as well as spontaneously occurring tumors from the larynx, pharynx, mammary gland, uterine cervix and vulva [91]. Moreover, TNF-α treatment sensitized tumors that initially did not respond to antibody treatment [91].

We aimed to find the underlying pathway responsible for the regulation of cytokines. We blocked the downstream pathway of EGFR by several inhibitors with or without the stimulation of IFNγ and TNFα and found that the inhibition of MEK1 and JNK significantly increased the cytokines at the gene level. A previous study showed

that activation of cells via IFNγR/TNFR results in cytokine mRNA production, but this mRNA is destabilized via EGFR-mediated overexpressed MEK/ERK1/2. Inhibition of ERK1/2 induces an even more severe inflammatory response in the skin [92], showing that EGFR and its downstream pathway suppresses the local immune response. Others have demonstrated that the MEK pathway selectively downregulates the human rhinovirus-16-induced epithelial production of CXCL10. Furthermore, PD98059 and U0126, two inhibitors of the MEK1/2-ERK MAPK pathway, significantly enhanced HRV-16-induced CXCL10 [93]. Our data presented in chapter 5 show that MEK1-inhibitor PD98059 alone did not alter *CCL5, CXCL9* and *CXCL10*, but when combined with IFNγ and TNFα significantly enhanced the gene expression of *CXCL9* and *CXCL10*, as well as *CCL5* in SCC4. Similar results were also observed after JNK inhibition by SP600125 when combined with IFNγ and TNFα stimulation. The JNK pathway plays a complex role in innate and adaptive immune systems. When MKP1 is knocked out, JNK signaling is activated, resulting in enhanced cytokine production of CCL2, CXCL10, TNF, IL6 and IL10, which leads to massive neutrophil infiltration to the lung and liver [94]. In Mkp5 knockout mice, activated JNK signaling increases TNF, IL6, IFNβ, IFNγ and TGFβ production by innate immune cells, and decreases Th1 and Th2 cytokines production by adaptive immune cells [95, 96]. Our data show that JNK inhibition combined with IFNγ and TNFα significantly increased *CCL5*, *CXCL9* and *CXCL10* gene expression, especially in the HPV+ OPSCC cell lines, while EGFR blocking enhanced *CCL5, CXCL9* and *CXCL10* production mainly via the downstream JNK and MEK1 pathways. Interestingly, EGFR blocking by cetuximab plus IFNγ and TNFα increased the T-cell-attracting chemokines but decreased IL1β expression. Tumor-derived IL1β secreted into the tumor microenvironment has been shown to induce the accumulation of MDSC possessing an enhanced capacity to suppress T cells [97]. Blocking of the downstream molecule c-RAF by GW5074 blunted the IFNγ and TNFα-induced expression of the T-cell-attracting chemokines while enhancing the expression of IL-1β, thereby confirming earlier reports that RAF can be activated by both IFNγ [98] and TNFα [99], and revealing an important role for c-RAF in

relaying the signals induced by IFNγ and TNFα that lead to cytokine production. Thus, EGFR blockade may stimulate the attraction of T cells while suppressing that of MDSC.

Besides the EGFR signaling pathway, many other oncogenic signaling pathways may also have an impact on immune signaling [100]. β-catenin-positive tumors had minimal T-cell infiltration due to the reduced production of CC-chemokine ligand 4 (CCL4) by tumor cells, resulting in a failure to recruit basic leucine zipper transcriptional factor ATF-like 3 lineage dendritic cells (BATF3 DCs) into the tumor microenvironment. Owing to a lack of CXCL10 production by BATF3 DCs, effector T cells are not recruited into the tumor [100, 101]. In addition, activation of MYC signaling enhances the expression of leukocyte surface antigen CD47 and PD-L1 on the tumor, thus interfering with antigen uptake by antigen-presenting cells (APCs) via engagement with signal-regulator protein-α (SIRPα) and inhibiting T-cell function via PD1 engagement, respectively [102]. Furthermore, loss of liver kinase B1 (LKB1) signaling within tumor cells results in increased expression of various cytokines, contributing to reduced T-cell infiltration and promotion of T-cell dysfunction [103]. Loss of PTEN protein function activates PI3K, thereby inhibiting autophagy in tumor cells [104, 105], which diminishes T-cell priming and also mediates resistance to T-cell-mediated apoptosis [106-108]. Finally, TP53-mutated tumor cells lack production of key chemokines required for the recruitment of NK cells to the tumor microenvironment [109, 110]. Moreover, by using the pharmacological p53 activator nutlin-3a, local p53 activation reversed immunosuppression in the tumor microenvironment and induced tumor immunogenic cell death, leading to activation and expansion of polyfunctional $CD8^+$ CTLs and tumor regression. P53 activation only enhanced the antitumor response when the tumor microenvironment already comprised tumor-infiltrating leukocytes [111], suggesting that, similar to our findings with respect to EGFR blockade, p53 activation can amplify the local immune response.

5. Overall summary

In summary, we focused on the resistance of HPV-infected cells and HPV-related cancers to Th1 immunity. In HPV+ KCs, human papillomavirus impaired necroptosis by downregulating the key component of necroptosis RIPK3 through histone methylation. The global histone methyltransferase inhibitor DZNeP restored the expression of RIPK3 and thus enhanced Th1-cytokine-induced necroptosis. Human papillomavirus also made KC resistant to the antiproliferative effects of IFNγ by downregulating *IFITM1* and *RARRES1*, which are the antiproliferation genes. With respect to the Th1 immune response itself and the resistance to it in HPV-related cancer, we found that HPV-positive OPSCC was infiltrated with type 1 T cells, and if so, these patients displayed a far better survival when compared to the HPV-negative OPSCC. We found the presence of Th1 and Th17 cytokines, mainly IFNγ and TNFα, in the culture of TILs from HPV-positive OPSCC. IFNγ and TNFα can induce cell-growth arrest in OPSCC cell lines by upregulating the antiproliferation genes *IFITM1* and *RARRES1*. However, OPSCC cancer cell lines also display other mechanisms by which to escape the immune control of type 1 cytokines. Similar to HPV-positive KCs, the HPV-positive OPSCC cell lines lacked the expression of RIPK3 and were resistant to necroptosis induced by IFNγ and TNFα. In addition, our previous study showed that HPV+ KCs expressed high levels of EGFR and when this receptor was blocked by cetuximab it led to a decreased expression of IFRD1, resulting in increased NFkB/RelA K310 acetylation, and as a consequence enhanced expression and production of proinflammatory cytokines and chemokines [84]. We now showed that the EGFR is also overexpressed at the cell surface of OPSCC cell lines and that EFGR-signaling impaired the production of the T-cell-attracting cytokines CCL5, CXCL9 and CXCL10 when these cells are stimulated with the Th1 cytokines IFNγ and TNFα. We propose that this may prevent the start of an amplification cycle for the migration of T cells to the tumor environment. In contrast to our observations in KCs, the inhibition of cytokines by EGFR signaling resulted mostly from the activation of the downstream JNK and MEK pathways, albeit

that in one cell line a role for IFRD1 was found. Others found that overexpression of EGFR also induced the expression of PD-L1 while lowering that of MHC classes I and II [112-114]. This suggests that EGFR overexpression impairs both the attraction and function of T cells. Our previous study shows that the presence of intratumoral HPV16-specific T cells is important in controlling the disease progression and clinical outcomes [12-14], which makes it important to boost the HPV-specific type 1 T-cell response by vaccines [7-12]. However, due to the resistant mechanisms to immune control of HPV related OPSCC, a combination with other therapies is required. We show that cisplatin combined with TNFα was most effective in inducing apoptosis in OPSCC cell lines in vitro. Based on these results, showing that RIPK3 was absent in HPV-positive OPSCC because of DNA methylation, co-treatment of methylation inhibitor 5-AAD and caspase-8 inhibitor may have therapeutic effects on HPV related cancer. 5-AAD leads to increased T-cell recognition of tumor cells without influencing the proliferation and function of $CD4^+$ and $CD8^+$ T cells [115], and may increase the expression of RIPK3, followed by the inhibition of caspase-8 priming for necroptosis, whereby type 1 cytokines IFNγ and TNFα may consequently increase the necroptosis of OPSCC. However, both the pan-caspase inhibitor zVAD-FMK and the caspase-8 inhibitor z-IETD-FMK suppress human T-cell proliferation [81]. Ways of inhibiting caspase-8 without influencing T-cell proliferation are worthwhile to explore and a combination with adoptive T-cell therapy could potentially be tested. We also found that the EGFR-inhibitor cetuximab combined with IFNγ and TNFα increased the production of the T-cell-attracting cytokines CCL5, CXCL9 and CXCL10, which resulted in the increased migration of $CD4^+$ and $CD8^+$ T cells in vitro. The downstream of the EGFR, JNK and MEK1 pathways are mainly responsible for suppressing the production of *CCL5, CXCL9* and *CXCL10*. In vivo, EGFR-signaling blockade increased CCL2, CCL5 and CXCL10 in KCs, and in a mouse model, the use of a selective EGFR kinase blocker resulted in a markedly enhanced immune response with increased chemokine expression and a more dense inflammatory cell infiltrate in the skin [116]. This provides evidence that the blockade of EGFR may also increase tumor-

infiltrated immune cells *in vivo*. In all, this thesis presents the mechanisms of Th1 immune regulation in HPV and HPV-related head and neck cancer.

Reference

1. Moscicki A-B. Impact of HPV infection in adolescent populations. Journal of Adolescent Health. 2005;37(6):S3-S9.

2. Sasagawa T, Takagi H, Makinoda S. Immune responses against human papillomavirus (HPV) infection and evasion of host defense in cervical cancer. Journal of Infection and Chemotherapy. 2012;18(6):807-15.

3. Papasavvas E, Surrey LF, Glencross DK, Azzoni L, Joseph J, Omar T, et al. High-risk oncogenic HPV genotype infection associates with increased immune activation and T cell exhaustion in ART-suppressed HIV-1-infected women. OncoImmunology. 2016;5(5):e1128612.

4. Westrich JA, Warren CJ, Pyeon D. Evasion of host immune defenses by human papillomavirus. Virus research. 2017;231:21-33.

5. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Research. 2002;62(2):472-9.

6. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer research. 2003;63(3):636-41.

7. Kim TJ, Jin H-T, Hur S-Y, Yang HG, Seo YB, Hong SR, et al. Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. Nature communications. 2014;5:5317.

8. Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, Lee JC, et al. Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. Science translational medicine. 2012;4(155):155ra38-ra38.

9. Trimble CL, Morrow MP, Kraynyak KA, Shen X, Dallas M, Yan J, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. The Lancet. 2015;386(10008):2078-88.

10. Kenter GG, Welters MJ, Valentijn ARP, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. New England Journal of Medicine. 2009;361(19):1838-47.

11. van Poelgeest MI, Welters MJ, Vermeij R, Stynenbosch LF, Loof NM, Berends-van der Meer DM, et al. Vaccination against oncoproteins of HPV16 for noninvasive vulvar/vaginal lesions: lesion clearance is related to the strength of the T-cell response. Clinical Cancer Research. 2016;22(10):2342-50.

12. Welters MJ, Kenter GG, van Steenwijk PJdV, Löwik MJ, Berends-van der Meer DM, Essahsah F, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proceedings of the National Academy of Sciences. 2010;107(26):11895-9.

13. Fridman WH, Zitvogel L, Sautès–Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. Nature reviews Clinical oncology. 2017;14(12):717.

14. Welters MJ, Ma W, Santegoets SJ, Goedemans R, Ehsan I, Jordanova ES, et al. Intratumoral HPV16-specific T-cells constitute a type 1 oriented tumor microenvironment to improve survival in HPV16-driven oropharyngeal cancer. Clinical Cancer Research. 2017:clincanres. 2140.017.

15. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990;63(6):1129-36.

16. Hengstermann A, D'silva MA, Kuballa P, Butz K, Hoppe-Seyler F, Scheffner M. Growth suppression induced by downregulation of E6-AP expression in human papillomavirus-positive cancer cell lines depends on p53. Journal of virology. 2005;79(14):9296-300.

17. Münger K, Werness B, Dyson N, Phelps W, Harlow E, Howley P. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. The EMBO journal. 1989;8(13):4099-105.

18. Wells SI, Francis DA, Karpova AY, Dowhanick JJ, Benson JD, Howley PM. Papillomavirus E2 induces senescence in HPV-positive cells via pRB-and p21CIP-dependent pathways. The EMBO journal. 2000;19(21):5762-71.

19. Webster K, Parish J, Pandya M, Stern PL, Clarke AR, Gaston K. The human papillomavirus (HPV) 16 E2 protein induces apoptosis in the absence of other HPV proteins and via a p53-dependent pathway. Journal of Biological Chemistry. 2000;275(1):87-94.

20. Bellanger S, Tan CL, Xue YZ, Teissier S, Thierry F. Tumor suppressor or oncogene? A critical role of the human papillomavirus (HPV) E2 protein in cervical cancer progression. American journal of cancer research. 2011;1(3):373.

21. Ning Y, Riggins RB, Mulla JE, Chung H, Zwart A, Clarke R. IFNγ restores breast cancer sensitivity to fulvestrant by regulating STAT1, IFN regulatory factor 1, NF-κB, BCL2 family members, and signaling to caspase-dependent apoptosis. Molecular cancer therapeutics. 2010;9(5):1274-85.

22. Rakshit S, Chandrasekar BS, Saha B, Victor ES, Majumdar S, Nandi D. Interferon-gamma induced cell death: Regulation and contributions of nitric oxide, cJun N-terminal kinase, reactive oxygen species and peroxynitrite. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2014;1843(11):2645-61.

23. Li P, Du Q, Cao Z, Guo Z, Evankovich J, Yan W, et al. Interferon-gamma induces autophagy with growth inhibition and cell death in human hepatocellular carcinoma (HCC) cells through interferon-regulatory factor-1 (IRF-1). Cancer letters. 2012;314(2):213-22.

24. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. Journal of leukocyte biology. 2004;75(2):163-89.

25. Hancock GE, Kaplan G, Cohn ZA. Keratinocyte growth regulation by the products of immune cells. Journal of Experimental Medicine. 1988;168(4):1395-402.

26. Saunders NA, Jetten AM. Control of growth regulatory and differentiation-specific genes in human epidermal keratinocytes by interferon gamma. Antagonism by retinoic acid and transforming growth factor beta 1. Journal of Biological Chemistry. 1994;269(3):2016-22.

27. Bosserhoff A, Kortylewski M, Komyod W, Kauffmann M-E, Heinrich PC, Behrmann I. Interferon-γ-mediated growth regulation of melanoma cells: involvement of STAT1-dependent and STAT1-independent signals. Journal of Investigative Dermatology. 2004;122(2):414-22.

28. Dimco G, Knight RA, Latchman DS, Stephanou A. STAT1 interacts directly with cyclin D1/Cdk4 and mediates cell cycle arrest. Cell cycle. 2010;9(23):4638-49.

29. Raveh T, Hovanessian AG, Meurs EF, Sonenberg N, Kimchi A. Double-stranded RNA-dependent protein kinase mediates c-Myc suppression induced by type I interferons. Journal of Biological Chemistry. 1996;271(41):25479-84.

30. Hong S, Mehta KP, Laimins LA. Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance. Journal of virology. 2011;85(18):9486-94.

31. Yang G, Xu Y, Chen X, Hu G. IFITM1 plays an essential role in the antiproliferative action of interferon-γ. Oncogene. 2007;26(4):594.

32. Youssef EM, Chen X-q, Higuchi E, Kondo Y, Garcia-Manero G, Lotan R, et al. Hypermethylation and silencing of the putative tumor suppressor Tazarotene-induced gene 1 in human cancers. Cancer research. 2004;64(7):2411-7.

33. Mizuiri H, Yoshida K, Toge T, Oue N, Aung PP, Noguchi T, et al. DNA methylation of genes linked to retinoid signaling in squamous cell carcinoma of the esophagus: DNA methylation of CRBP1 and TIG1 is associated with tumor stage. Cancer science. 2005;96(9):571-7.

34. Yanatatsaneejit P, Chalermchai T, Kerekhanjanarong V, Shotelersuk K, Supiyaphun P, Mutirangura A, et al. Promoter hypermethylation of CCNA1,

RARRES1, and HRASLS3 in nasopharyngeal carcinoma. Oral oncology. 2008;44(4):400-6.

35. Wu C-C, Shyu R-Y, Chou J-M, Jao S-W, Chao P-C, Kang J-C, et al. RARRES1 expression is significantly related to tumour differentiation and staging in colorectal adenocarcinoma. European Journal of Cancer. 2006;42(4):557-65.

36. Takai N, Kawamata N, Walsh CS, Gery S, Desmond JC, Whittaker S, et al. Discovery of epigenetically masked tumor suppressor genes in endometrial cancer. Molecular cancer research. 2005;3(5):261-9.

37. Jing C, El-Ghany MA, Beesley C, Foster CS, Rudland PS, Smith P, et al. Tazarotene-induced gene 1 (TIG1) expression in prostate carcinomas and its relationship to tumorigenicity. Journal of the National Cancer Institute. 2002;94(7):482-90.

38. Branca M, Ciotti M, Giorgi C, Santini D, Di Bonito L, Costa S, et al. Up-regulation of proliferating cell nuclear antigen (PCNA) is closely associated with high-risk human papillomavirus (HPV) and progression of cervical intraepithelial neoplasia (CIN), but does not predict disease outcome in cervical cancer. European Journal of Obstetrics and Gynecology and Reproductive Biology. 2007;130(2):223-31.

39. Bologna-Molina R, Mosqueda-Taylor A, Molina-Frechero N, Mori-Estevez AD, Sánchez-Acuña G. Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumor. Medicina oral, patologia oral y cirugia bucal. 2013;18(2):e174.

40. Galluzzi L, Vitale I, Abrams J, Alnemri E, Baehrecke E, Blagosklonny M, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell death and differentiation. 2012;19(1):107.

41. Orzalli MH, Kagan JC. Apoptosis and Necroptosis as Host Defense Strategies to Prevent Viral Infection. Trends in cell biology. 2017.

42. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell host & microbe. 2012;11(3):290-7.

43. Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. Cell host & microbe. 2010;7(4):302-13.

44. Thapa RJ, Ingram JP, Ragan KB, Nogusa S, Boyd DF, Benitez AA, et al. DAI senses influenza A virus genomic RNA and activates RIPK3-dependent cell death. Cell host & microbe. 2016;20(5):674-81.

45. Kuriakose T, Man SM, Malireddi RS, Karki R, Kesavardhana S, Place DE, et al. ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. Science immunology. 2016;1(2).

46. Nogusa S, Thapa RJ, Dillon CP, Liedmann S, Oguin TH, Ingram JP, et al. RIPK3 activates parallel pathways of MLKL-driven necroptosis and FADD-mediated apoptosis to protect against influenza A virus. Cell host & microbe. 2016;20(1):13-24.

47. Berger AK, Danthi P. Reovirus activates a caspase-independent cell death pathway. MBio. 2013;4(3):e00178-13.

48. Guo H, Omoto S, Harris PA, Finger JN, Bertin J, Gough PJ, et al. Herpes simplex virus suppresses necroptosis in human cells. Cell host & microbe. 2015;17(2):243-51.

49. Omoto S, Guo H, Talekar GR, Roback L, Kaiser WJ, Mocarski ES. Suppression of RIP3-dependent necroptosis by human cytomegalovirus. Journal of biological chemistry. 2015;290(18):11635-48.

50. Kwok C, Pavlosky A, Lian D, Jiang J, Huang X, Yin Z, et al. Necroptosis Is Involved in CD4+ T Cell-Mediated Microvascular Endothelial Cell Death and Chronic Cardiac Allograft Rejection. Transplantation. 2017;101(9):2026-37.

51. Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. Nature. 2015;517(7534):311.

52. Moriwaki K, Chan FK-M. RIP3: a molecular switch for necrosis and inflammation. Genes & development. 2013;27(15):1640-9.

53. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annual review of immunology. 2009;27:519-50.

54. Niebler M, Qian X, Höfler D, Kogosov V, Kaewprag J, Kaufmann AM, et al. Post-translational control of IL-1β via the human papillomavirus type 16 E6 oncoprotein: a novel mechanism of innate immune escape mediated by the E3-ubiquitin ligase E6-AP and p53. PLoS pathogens. 2013;9(8):e1003536.

55. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. New England Journal of Medicine. 2010;363(1):24-35.

56. Wansom D, Light E, Worden F, Prince M, Urba S, Chepeha DB, et al. Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. Archives of Otolaryngology–Head & Neck Surgery. 2010;136(12):1267-73.

57. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay NEH, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clinical cancer research. 2006;12(2):465-72.

58. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, et al. PD-1–expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. Cancer research. 2013;73(1):128-38.

59. Vince JE, Pantaki D, Feltham R, Mace PD, Cordier SM, Schmukle AC, et al. TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (TNF) to efficiently activate NF-κB and to prevent TNF-induced apoptosis. Journal of Biological Chemistry. 2009;284(51):35906-15.

60. Shen RR, Zhou AY, Kim E, O'Connell JT, Hagerstrand D, Beroukhim R, et al. TRAF2 is an NF-κB-activating oncogene in epithelial cancers. Oncogene. 2015;34(2):209.

61. Imoto I, Yang Z-Q, Pimkhaokham A, Tsuda H, Shimada Y, Imamura M, et al. Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. Cancer research. 2001;61(18):6629-34.

62. Zhang R, Liu Y, Hammache K, He L, Zhu B, Cheng W, et al. The role of FADD in pancreatic cancer cell proliferation and drug resistance. Oncology letters. 2017;13(3):1899-904.

63. Vandenabeele P, Galluzzi L, Berghe TV, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature reviews Molecular cell biology. 2010;11(10):700.

64. Vanlangenakker N, Berghe TV, Vandenabeele P. Many stimuli pull the necrotic trigger, an overview. Cell death and differentiation. 2012;19(1):75.

65. Wu W, Liu P, Li J. Necroptosis: an emerging form of programmed cell death. Critical reviews in oncology/hematology. 2012;82(3):249-58.

66. Vanlangenakker N, Bertrand M, Bogaert P, Vandenabeele P, Berghe TV. TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members. Cell death & disease. 2011;2(11):e230.

67. Fukasawa M, Kimura M, Morita S, Matsubara K, Yamanaka S, Endo C, et al. Microarray analysis of promoter methylation in lung cancers. Journal of human genetics. 2006;51(4):368-74.

68. Koo G-B, Morgan MJ, Lee D-G, Kim W-J, Yoon J-H, Koo JS, et al. Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in response to chemotherapeutics. Cell research. 2015;25(6):707.

69. Su Z, Yang Z, Xie L, DeWitt J, Chen Y. Cancer therapy in the necroptosis era. Cell death and differentiation. 2016;23(5):748.

70. Moriwaki K, Bertin J, Gough P, Orlowski G, Chan FK. Differential roles of RIPK1 and RIPK3 in TNF-induced necroptosis and chemotherapeutic agent-induced cell death. Cell death & disease. 2016;6(2):e1636.

71. Schmidt SV, Seibert S, Walch-Rückheim B, Vicinus B, Kamionka E-M, Pahne-Zeppenfeld J, et al. RIPK3 expression in cervical cancer cells is required for PolyIC-induced necroptosis, IL-1α release, and efficient paracrine dendritic cell activation. Oncotarget. 2015;6(11):8635.

72. Nugues A-L, El Bouazzati H, Hetuin D, Berthon C, Loyens A, Bertrand E, et al. RIP3 is downregulated in human myeloid leukemia cells and modulates apoptosis and caspase-mediated p65/RelA cleavage. Cell death & disease. 2014;5(8):e1384.

73. Liu P, Xu B, Shen W, Zhu H, Wu W, Fu Y, et al. Dysregulation of TNFα-induced necroptotic signaling in chronic lymphocytic leukemia: suppression of CYLD gene by LEF1. Leukemia. 2012;26(6):1293.

74. Sun L, Wang H, Wang Z, He S, Chen S, Liao D, et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell. 2012;148(1):213-27.

75. Colbert LE, Fisher SB, Hardy CW, Hall WA, Saka B, Shelton JW, et al. Pronecrotic mixed lineage kinase domain-like protein expression is a prognostic

biomarker in patients with early-stage resected pancreatic adenocarcinoma. Cancer. 2013;119(17):3148-55.

76. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell. 2011;144(5):646-74.

77. Han W, Li L, Qiu S, Lu Q, Pan Q, Gu Y, et al. Shikonin circumvents cancer drug resistance by induction of a necroptotic death. Molecular cancer therapeutics. 2007;6(5):1641-9.

78. Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK, et al. Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. The Journal of clinical investigation. 2010;120(4):1310-23.

79. McCabe K, Bacos K, Lu D, Delaney J, Axelrod J, Potter M, et al. Triggering necroptosis in cisplatin and IAP antagonist-resistant ovarian carcinoma. Cell death & disease. 2014;5(10):e1496.

80. Rajah T, Chow S. The inhibition of human T cell proliferation by the caspase inhibitor z-VAD-FMK is mediated through oxidative stress. Toxicology and applied pharmacology. 2014;278(2):100-6.

81. Lawrence C, Chow S. Suppression of human T cell proliferation by the caspase inhibitors, z-VAD-FMK and z-IETD-FMK is independent of their caspase inhibition properties. Toxicology and applied pharmacology. 2012;265(1):103-12.

82. van der Sluis TC, van Duikeren S, Huppelschoten S, Jordanova ES, Nejad EB, Sloots A, et al. Vaccine-induced tumor necrosis factor–producing T cells synergize with cisplatin to promote tumor cell death. Clinical Cancer Research. 2015;21(4):781-94.

83. Cao W, Chi W-H, Wang J, Tang J-J, Lu Y-J. TNF-α promotes Doxorubicin-induced cell apoptosis and anti-cancer effect through downregulation of p21 in p53-deficient tumor cells. Biochemical and biophysical research communications. 2005;330(4):1034-40.

84. Tummers B, Goedemans R, Pelascini LP, Jordanova ES, Van Esch EM, Meyers C, et al. The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NFκB activation. Nature communications. 2015;6:6537.

85. Maiti GP, Mondal P, Mukherjee N, Ghosh A, Ghosh S, Dey S, et al. Overexpression of EGFR in head and neck squamous cell carcinoma is associated with inactivation of SH3GL2 and CDC25A genes. PloS one. 2013;8(5):e63440.

86. Dong Z-Y, Zhang J-T, Liu S-Y, Su J, Zhang C, Xie Z, et al. EGFR mutation correlates with uninflamed phenotype and weak immunogenicity, causing impaired response to PD-1 blockade in non-small cell lung cancer. OncoImmunology. 2017;6(11):e1356145.

87. Pollack BP, Sapkota B, Cartee TV. Epidermal growth factor receptor inhibition augments the expression of MHC class I and II genes. Clinical Cancer Research. 2011;17(13):4400-13.

88. Concha-Benavente F, Srivastava RM, Trivedi S, Lei Y, Chandran U, Seethala RR, et al. Identification of the cell-intrinsic and-extrinsic pathways downstream of EGFR and IFNγ that induce PD-L1 expression in head and neck cancer. Cancer research. 2016;76(5):1031-43.

89. Akbay EA, Koyama S, Carretero J, Altabef A, Tchaicha JH, Christensen CL, et al. Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. Cancer discovery. 2013;3(12):1355-63.

90. Garrido G, Lorenzano P, Sánchez B, Beausoleil I, Alonso DF, Pérez R, et al. T cells are crucial for the anti-metastatic effect of anti-epidermal growth factor receptor antibodies. Cancer Immunology, Immunotherapy. 2007;56(11):1701-10.

91. Hambek M, Solbach C, Schnuerch H-G, Roller M, Stegmueller M, Sterner-Kock A, et al. Tumor necrosis factor α sensitizes low epidermal growth factor receptor (EGFR)-expressing carcinomas for anti-EGFR therapy. Cancer research. 2001;61(3):1045-9.

92. Pastore S, Mascia F, Mariotti F, Dattilo C, Mariani V, Girolomoni G. ERK1/2 regulates epidermal chemokine expression and skin inflammation. The Journal of Immunology. 2005;174(8):5047-56.

93. Zaheer RS, Koetzler R, Holden NS, Wiehler S, Proud D. Selective transcriptional down-regulation of human rhinovirus-induced production of CXCL10 from airway epithelial cells via the MEK1 pathway. The Journal of Immunology. 2009;182(8):4854-64.

94. Wang X, Meng X, Kuhlman JR, Nelin LD, Nicol KK, English BK, et al. Knockout of Mkp-1 enhances the host inflammatory responses to gram-positive bacteria. The Journal of Immunology. 2007;178(8):5312-20.

95. Liu Y, Shepherd EG, Nelin LD. MAPK phosphatases—regulating the immune response. Nature Reviews Immunology. 2007;7(3):202.

96. Zhang Y, Blattman JN, Kennedy NJ, Duong J, Nguyen T, Wang Y, et al. Regulation of innate and adaptive immune responses by MAP kinase phosphatase 5. Nature. 2004;430(7001):793.

97. Elkabets M, Ribeiro VS, Dinarello CA, Ostrand-Rosenberg S, Di Santo JP, Apte RN, et al. IL-1β regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function. European journal of immunology. 2010;40(12):3347-57.

98. Zibara K, Zeidan A, Bjeije H, Kassem N, Badran B, El-Zein N. ROS mediates interferon gamma induced phosphorylation of Src, through the Raf/ERK pathway, in MCF-7 human breast cancer cell line. Journal of cell communication and signaling. 2017;11(1):57-67.

99. Modur V, Zimmerman GA, Prescott SM, McIntyre TM. Endothelial Cell Inflammatory Responses to Tumor Necrosis Factor α Ceramide-dependent and-independent Mitogen-activated Protein Kinase Cascades. Journal of Biological Chemistry. 1996;271(22):13094-102.

100. Spranger S, Gajewski TF. Impact of oncogenic pathways on evasion of antitumour immune responses. Nature Reviews Cancer. 2018.

101. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. Nature. 2015;523(7559):231.

102. Casey SC, Tong L, Li Y, Do R, Walz S, Fitzgerald KN, et al. MYC regulates the antitumor immune response through CD47 and PD-L1. Science. 2016:aac9935.

103. Koyama S, Akbay EA, Li YY, Aref AR, Skoulidis F, Herter-Sprie GS, et al. STK11/LKB1 deficiency promotes neutrophil recruitment and proinflammatory cytokine production to suppress T-cell activity in the lung tumor microenvironment. Cancer research. 2016;76(5):999-1008.

104. Peng W, Chen JQ, Liu C, Malu S, Creasy C, Tetzlaff MT, et al. Loss of PTEN promotes resistance to T cell–mediated immunotherapy. Cancer discovery. 2016;6(2):202-16.

105. Sai J, Owens P, Novitskiy SV, Hawkins OE, Vilgelm AE, Yang J, et al. PI3K inhibition reduces mammary tumor growth and facilitates antitumor immunity and anti-PD1 responses. Clinical Cancer Research. 2017;23(13):3371-84.

106. Page DB, Hulett TW, Hilton TL, Hu H-M, Urba WJ, Fox BA. Glimpse into the future: harnessing autophagy to promote anti-tumor immunity with the DRibbles vaccine. Journal for immunotherapy of cancer. 2016;4(1):25.

107. Li Y, Wang L-x, Pang P, Twitty C, Fox BA, Aung S, et al. Cross-presentation of tumor associated antigens through tumor-derived autophagosomes. Autophagy. 2009;5(4):576-7.

108. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, et al. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science. 2011;334(6062):1573-7.

109. Iannello A, Thompson TW, Ardolino M, Lowe SW, Raulet DH. p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells. Journal of Experimental Medicine. 2013:jem. 20130783.

110. Quigley D, Silwal-Pandit L, Dannenfelser R, Langerød A, Vollan HKM, Vaske C, et al. Lymphocyte invasion in IC10/basal-like breast tumors is associated with wild-type TP53. Molecular Cancer Research. 2015;13(3):493-501.

111. Guo G, Yu M, Xiao W, Celis E, Cui Y. Local activation of p53 in the tumor microenvironment overcomes immune suppression and enhances antitumor immunity. Cancer research. 2017;77(9):2292-305.

112. Concha-Benavente F, Srivastava RM, Trivedi S, Lei Y, Chandran U, Seethala RR, et al. Identification of the cell-intrinsic and extrinsic pathways downstream of EGFR and IFNγ that induce PD-L1 expression in head and neck cancer. Cancer research. 2015:canres. 2001.15.

113. Srivastava RM, Trivedi S, Concha-Benavente F, Jie H-B, Wang L, Seethala RR, et al. STAT1 induced HLA class I upregulation enhances immunogenicity and clinical response to anti-EGFR mAb cetuximab therapy in HNC patients. Cancer immunology research. 2015:canimm. 0053.2015.

114. Li X, Lian Z, Wang S, Xing L, Yu J. Interactions between EGFR and PD-1/PD-L1 pathway: Implications for treatment of NSCLC. Cancer letters. 2018;418:1-9.

115. Gang A, Frøsig T, Brimnes M, Lyngaa R, Treppendahl M, Grønbæk K, et al. 5-Azacytidine treatment sensitizes tumor cells to T-cell mediated cytotoxicity and modulates NK cells in patients with myeloid malignancies. Blood cancer journal. 2014;4(3):e197.

116. Mascia F, Mariani V, Girolomoni G, Pastore S. Blockade of the EGF receptor induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation. The American journal of pathology. 2003;163(1):303-12.

SUMMARY

The clearance of high-risk human papillomavirus (hrHPV) infected or transformed cells requires the local presence of a strong type 1 T cell response but HPV has evolved mechanisms to resist immune attack. The Th1 cytokines IFNγ and TNFα can induce programmed necrosis (necroptosis), which is one of the mechanisms to amplify the immune response and thereby to resist viral infections. We found that hrHPV impaired IFNγ and TNFα-induced necroptosis by down-regulation the expression of RIPK3, a key component in the necroptosis pathway, in HPV+ keratinocytes. The mechanism which was responsible for down-regulating RIPK3 expression was due to HPV-induced histone methylation. The methyltransferase inhibitor DZNeP restored the expression of RIPK3 in HPV+ keratinocytes, and increased necroptosis in HPV+ keratinocytes. The Th1 cytokines IFNγ and TNFα can also mediate the growth arrest of infected keratincoytes, but hrHPV also effectively inhibited the arrest of cell growth by down-regulating IFITM1. Thus, we identified two mechanisms that may explain how the human papillomavirus itself can thrive for a long time in an immune competent host.

We also studied how HPV-induced cancers may resist the immune system. We showed that tumor infiltrating IFNγ and TNFα producing HPV16 specific T cells were present in about 65% of all HPV-associated head and neck cancers and this correlated strongly with improved survival of patients after treatment. Yet, despite the presence of such T cells in the majority of cancers, before treatment the tumor still progressed pointing at resistance mechanisms. We found that HPV16-positive tumor cells also lacked expression of RIPK3 and were not sensitive to the necroptosis induced by IFNγ and TNFα. However, this resistance could be overcome by treatment of these cancer cells with a combination of cisplatin and these Th1 cytokines, resulting in enhanced killing of tumor cells. Last but not least we revealed another possible resistance mechanism in head and neck cancer. We showed that treatment with a combination IFNγ and TNFα and the clinically applied EGFR inhibitor Cetuximab resulted in an increased gene expression of multiple cytokines, including CXCL9,

CXCL10 and CCL5, which may amplify the attraction of more lymphocytes to the tumor site. Hence, EGFR signaling in tumors dampens inflammation. We revealed that the molecules JNK and MEK1, downstream of the EGFR, played a major role in mediating the suppression of IFNγ and TNFα-mediated production of CCL5, CXCL9 and CXCL10. In addition, we found that c-RAF signaling was important for the production of CCL5, CXCL9 and CXCL10 in head and neck cancer cells.

Overall, we showed the mechanisms how Th1 immune response regulate hrHPV infection and hpv related cancer, and we also showed that hrHPV-infected and –transformed cells developed several means to dampen and/or resist the effects of Th1 immune response.

SAMENVATTING

Een sterke type 1 T-cel reactie is nodig om hoog-risico humaan papillomavirus (hrHPV)-geïnfecteerde of -getransformeerde keratinocyten te klaren. Echter hrHPV is in staat om de aanval van het afweersysteem te weerstaan. De type 1 cytokinen IFNγ en TNFα, die geproduceerd worden door de T-cellen, induceren geprogrammeerde celdood middels necrose (necroptose). Een mechanisme waardoor de afweerreactie tegen virussen en kanker versterkt kan worden. In onze studies vonden we dat hrHPV de door IFNγ- en TNFα-geïnduceerde necroptose reduceerde door in de geïnfecteerde cellen de expressie van het sleuteleiwit RIPK3 te verminderen d.m.v. methylatie van histonen. Het gebruik van de methyltransferaseremmer DZNeP herstelde de expressie van RIPK3 en verhoogde de IFNγ en TNFα geïnduceerde necroptose in de geïnfecteerde cellen. Daarnaast remmen de type 1 cytokinen IFNγ en TNFα ook de groei van geïnfecteerde keratinocyten waardoor er minder virus geproduceerd kan worden. Echter hrHPV was ook in staat om dit proces te ondermijnen door de expressie van IFITM1 te verminderen. Er werden dus twee mechanismen geïdentificeerd die kunnen verklaren waarom hrHPV in staat is om langdurig in een gezond individu aanwezig te zijn.

We hebben ook bestudeerd hoe hrHPV-veroorzaakte kanker het afweersysteem kan weerstaan. Onze studies toonde aan dat in ongeveer 65% van de door hrHPV-veroorzaakte hoofd-hals kankers HPV-specifieke type 1 T-cellen werden aangetroffen. De aanwezigheid van deze cellen correleerde sterk met een verbeterde overleving van de patiënten na hun behandeling. Echter zonder behandeling en ondanks de aanwezigheid van de HPV-specifieke T-cellen, groeide de tumor gewoon door. Dit duidt erop dat ook in de hrHPV-geïnduceerde tumoren ontsnappingsmechanismen aanwezig zijn. Wij vonden ook in hrHPV-positieve tumoren dat het eiwit RIPK3 afwezig was en dat deze cellen niet gevoelig waren voor IFNγ- en TNFα-geïnduceerde necroptose. Echter, door deze tumor cellen niet alleen te behandelen met de twee cytokinen maar ook met het

chemotherapeuticum cisplatinum, konden de tumorcellen toch gedood worden.

Daarnaast vonden we dat er mogelijk ook nog een ander ontsnappingsmechanisme een rol kan spelen in hoofd-hals kanker. Behandeling van verschillende hoofd-hals kanker cellijnen met een combinatie van de twee type 1 cytokinen en het in de kliniek gebruikte antilichaam cetuximab, een EGFR remmer, resulteerde in de verhoogde genexpressie van verschillende cytokinen, waaronder CXCL9, CXCL10 and CCL5. De productie van deze cytokinen kan het rekruteren van witte bloedcellen, zoals de T-cellen, naar de tumor versterken. Dit toonde aan dat de constitutieve EGFR signalering in de tumorcellen lokale ontsteking remt. Verder onderzoek liet zien dat de twee moleculen JNK en MEK1, die door EGFR geactiveerd kunnen worden, een belangrijke rol spelen in de remming van de IFNγ en TNFα- geïnduceerde productie van CCL5, CXCL9 en CXCL10. Verder onthulde onze studie ook dat het signaalmolecuul c-RAF belangrijk was voor de IFNγ en TNFα- gestimuleerde productie van CCL5, CXCL9 en CXCL10 in de hoofd-hals kankercellen.

CURRICULUM VITAE

Wenbo Ma was born in Lanzhou, Gansu province in China. He finished his bachelor in the subject of Medical Laboratory in the Second Clinical Medical College of Lanzhou University in China in 2010. Meanwhile Wenbo did the internship in the Clinical Laboratory in the Second Hospital of Lanzhou University since 2009 to 2010. After the bachelor education, from 2010 to 2013 Wenbo did his Master research in the subject of Biochemistry and Molecular Biology in the Department of Signal Transduction and Molecular Targeted Therapy, State Key Laboratory of Biotherapy, West China Medical Center of Sichuan University in China. Prof. Yangfu Jiang was his supervisor during the Master education. The project during his Master was up-regulation of heat shock protein 27 conferred resistance to Actinomycin D-induced apoptosis in cancer cells. Since 2013 to 2018, Wenbo did his PhD in the subject of Molecular Immunology in the Department of Medical Oncology in Leiden University Medical Center in Netherlands. His supervisor during his PhD was Prof. Sjoerd H van der Burg. His PhD project was Th1 immune regulation of HPV and HPV related cancer. His PhD research work has lead to this thesis.

PUBLICATIONS

1. Ma W, Concha-Benavente F, Santegoets SJ, Ehsan I, Welters MJP, Ferris RL, Van Der Burg SH: EGFR signaling suppresses type 1 cytokine-induced T-cell attracting chemokine secretion in head and neck cancer. PLoS One. 2018 Sep 7;13(9):e0203402

2. Welters MJ, Ma W, Santegoets SJ, Goedemans R, Ehsan I, Jordanova KS, van Ham VJ, van Unen V, Koning F, van Egmond SI, van der Burg SH: Intratumoral HPV16-specific T-cells constitute a type 1 oriented tumor microenvironment to improve survival in HPV16-driven oropharyngeal cancer. Clin Cancer Res. 2018 Feb 1;24(3):634-647

3. Ma W, Melief CJ, van der Burg SH: Control of immune escaped human papilloma virus is regained after therapeutic vaccination. Current Opinion in Virology 2017, 23:16-22.

4. Ma W, Tummers B, Van Esch EM, Goedemans R, Melief CJ, Meyers C, Boer JM, Van Der Burg SH: Human papillomavirus downregulates the expression of IFITM1 and RIPK3 to escape from IFNγ-and TNFα-mediated anti-proliferative effects and necroptosis. Front Immunol. 2016 Nov 22;7:496

5.Ma W, Teng Y, Hua H, Hou J, Luo T, Jiang Y: Upregulation of heat shock protein 27 confers resistance to actinomycin D-induced apoptosis in cancer cells. The FEBS journal 2013, 280:4612-4624.

ACKNOWLEDGMENT

I'm glad that I had the opportunity to do my PhD research in the Department of Medical Oncology in LUMC in Netherlands. During these years my PhD, I learned a lot from the weekly work discussion with Sjoerd. On the aspect of research, Sjoerd guided me from every aspect of research with patience. Your efficient way of solving problems was wonderful. At the beginning of my PhD, the language and culture difference was a big challenge for me. Thank Lien for giving me a lot of understanding and support in the lab. Thank Bart for showing me the techniques needed for my PhD research and leading me on the track of my study. Thank Bianca for showing me the efficient way of making protocols for the experiment. Also I want to thank Prof. Peter and Prof. Jochemsen from MCB department for the advice of Molecular Biology. Thank Marij for the collaboration of the project and the support during my first oral presentation on the EUROGIN 2016 Congress in Salzburg. Thank Ilina for helping me from some large experiments. Thank Saskia for guiding me of FACS with the very detailed and accurate protocol. I also want to thank all the other colleagues in Medical Oncology.

I also want to thank all my friends during these years. Eleni, we started our PhD almost at the same time, thank you for all the support and understanding throughout my whole PhD. Thank Jia Liu for all the help when I just arrived in Netherlands and support during these years. Thank Rui Zhang, Guangsheng Du, Puning Liu, Yingguang Li for your accompany. The time I spent with you guys was the happiest time during these years. I also want to thank Yifei Bi, Jing Niu, Zhenni Li, Junling He, Ka Zhang, Quanchi Chen, Yali Wang, Yanming Guo, thank you for all the wonderful memories!

In the end, I want to thank my family. The love, support, understanding from my parents and grandparents are always the treasures in my heart. No matter where I go and no matter when it is, home is always the shelter of me!

在荷兰的这几年里，我最想感谢我的朋友们，我最快乐的时光都是和你们在一起，我们一起去田间、海边、森林、大湖边骑行，我们一起旅游，以及每个周末在博士楼 132 的聚餐和谈心，都是我这几年里最美好的回忆。我们一起共享欢笑，分担痛苦，尤其是你们给我的支持和理解，给我了前行的力量和希望，祝福我的朋友们前程似锦，幸福安康！

我还想感谢我的父母和祖父母，他们给了我包容和爱，这些世间最平凡而微小的爱，却是面对黑暗最伟大的力量！祝福我和我的家人身体健康，平安幸福！