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The impact of human papillomavirus-mediated transformation on IRF7 expression in keratinocytes

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1. Summary

Persistent high-risk HPV infection is the major aetiological factor for the development of cervical cancer, since over 99% of the cervical cancer biopsy specimens are found to be positive for HPV DNA. Both genotypes HPV16 and HPV18 are together responsible for 70% of cervical carcinomas. IFNs are an important part of the host defense against viral infection. Numerous previous studies have reported that IFNβ can induce the IRF7 expression through the JAK-STAT signaling pathway. It has been proposed that oncoproteins of high-risk HPV can suppress type I IFN signaling.

The mechanisms by which HPV can modify the cellular response to the treatment with cytokines such as TNFα and IFNβ have been extensively studied.

In this study we examined the possible response of HPV-transformed (non-malignant as well as malignant cells) and normal human keratinocytes to the cytokines TNFα and IFNβ. Major interest was to explore differences in expression of the transcription factor IRF7. Quantitative RT-PCR was done to investigate the expression of four genes comprising IRF7, Mx1 as a further IFNβ-responsive gene, CCL20 as a further TNFα-responsive gene and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a house keeping gene.

We found that TNFα has limited ability to induce IRF7 expression in HPV-transformed and normal human keratinocytes. This induction is in some cases of HPV18-transformed keratinocytes stronger than in HPV-negative keratinocytes. In addition, our data show that IRF7 expression induced by exogenous IFNβ is retained in most HPV-positive malignant and non-malignant keratinocytes. Except HeLa cells, TNFα and IFNβ induce synergistically IRF7 expression in HPV-negative and HPV-transformed keratinocytes.

The present work demonstrates that the exogenous IFNβ signaling leading to induction of Mx1 expression and TNFα signaling leading to induction of CCL20 are not disturbed in HPV-transformed and normal human keratinocytes.

Thus, HPV16 and HPV18 may have no negative effects on the response of mucosal human keratinocytes to the treatment with TNFα and/or IFNβ.

2. Zusammenfassung

Eine persistierende Infektion mit Hochrisiko HPV stellt den wichtigsten ätiologischen Faktor für die Entstehung eines Zervixkarzinoms dar. In über 90% von zervikalen Gewebeproben kann HPV-DNA nachgewiesen werden. Die beiden Genotypen HPV16 und HPV18 sind zusammengenommen für 70% der Zervixkarzinome verantwortlich. Bei der Abwehrreaktion des Wirtsorganismus gegen virale Infektionen spielen Interferone eine wichtige Rolle. Zahlreiche frühere Studien konnten zeigen, dass IFNβ über den JAK-STAT Signalweg die Expression von IRF7 induzieren kann. Es wird vermutet, dass der Typ I IFN Signalweg durch Onkoproteine von Hochrisiko HPV unterdrückt werden kann. Der Mechanismus durch den HPV die zelluläre Reaktion nach Behandlung mit Zytokinen wie TNFα und IFNβ modifizieren können, ist intensiv untersucht worden.

In dieser Arbeit wurde die mögliche Reaktion von HPV-transformierten (nicht-malignen wie auch malignen Zellen) und normalen humanen Keratinozyten auf die Zytokine TNFα und IFNβ untersucht. Insbesondere sollten Unterschiede in der Expression des Transkriptionsfaktors IRF7 untersucht werden. Die Expression von den folgenden vier Genen, IRF7, Mx1 als ein weiteres IFNβ responsibles Gen, CCL20 als ein weiteres TNFα responsibles Gen sowie das Haushaltsgen Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) wurde mit Hilfe von quantitativer RT-PCR untersucht.

Es zeigte sich, dass die Expression von IRF7 in HPV-transformierten und normalen humanen Keratinozyten durch TNFα nur begrenzt induziert werden kann. Diese Induktion ist in HPV18-transformierten Keratinozyten in manchen Fällen stärker als in HPV-negativen Keratinozyten.

Weiterhin zeigen unsere Daten, dass die durch exogenes IFNβ induzierte Expression von IRF7 in den meisten HPV-positiven malignen und nicht malignen Keratinozyten beibehalten ist. Außer in HeLa Zellen lässt sich für TNFα und IFNβ

eine synergistische Induktion der Expression von IRF7 in HPV-negativen und HPV-transformierten Keratinozyten nachweisen.

Die vorliegende Arbeit zeigt, dass die exogene IFNβ Signaltransduktion, die zu einer Induktion der Expression von Mx1 führt und die TNFα Signaltransduktion, die zu einer Induktion von CCL20 führt, in HPV-transformierten und normalen humanen Keratinozyten nicht gestört ist.

Somit üben HPV16 und HPV18 vermutlich keinen negativen Effekt auf die Reaktion von mukosalen humanen Keratinozyten nach Behandlung mit TNFα und/oder IFNβ aus.

3. Glossary

BSA	Bovines Serum Albumin
C4 I	HPV18-positive human cervical carcinoma cell line
CaSki	HPV16-positive human cervical carcinoma cell line
CBP	CREP-binding protein
CCL20	CC chemokine ligand 20
cDNA	Complementary deoxyribonucleic acid
CIN	cervical intraepithelial neoplasia
CO_2	Carbon dioxide
Ct	Threshold cycle
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleolide triphosphate
DMEM	Dulbecco s modified eagle medium
DMSO	Dimethylsulfoxid
Ds	Double-strand
DTT	Dithiothreitol
E	Real-Time PCR efficiency
E1/2/4/5/6/7	Early proteins of HPV
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
et al	And others
EXLN	Primary exocervix keratinocytes
FCS	Fetal calf serum
Fig	Figure
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Hela	HPV18-positive human adenocarcinoma cell line
HPK1A	human foreskin keratinocytes immortalized with HPV16
HPV	Human papillomavirus

HPV16	HPV type 16
HPV18	HPV type 18
Hu	Human
IFN	Interferon
IFNβ	Interferon beta
IL	Interleukin
IRF	Interferon regulatory factor
ISGs	Interferon-stimulated genes
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK	Janus kinase
K51	human foreskin keratinocytes immortalized with HPV18
KBM	Keratinocyte cell basal medium
L	Late proteins of HPV
LARC	Liver and activation-regulated chemokine
LPS	Lipopolysaccaride
M	Milli
M	Molar
Mg	Milligram
$MgCl_2$	Magnesium chloride
MIP-3α	Macrophage inflammatory human protein-3 alpha
Ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
Mx1	Interferon-induced GTP-binding protein
MyD88	Myeloid differentiation factor 88
NFK13	Primary foreskin keratinocytes
NF-kB	Nuclear factor kB
ORF	Open-reading frame
PAMPs	Pathogen-associated molecular patterns

PBL	Peripheral blood lymphocytes
PBS	Phosphate-buffered salt solution
PCR	Polymerase chain reaction
P48	Protein 48
P53	Protein 53
P56	Protein 56
P107	Protein 107
P130	Protein 130
P300	Protein 300
R	Reporter
Rb	Retinoblastoma
RNA	Ribonucleic acid
Rpm	Revolution per minute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
SW756	HPV18-positive cells of a differentiated cervical squamous cell carcinoma
STAT	Signal transducer and activator of transcription
Q	Quencher
qPCR	Quantitive polymerase chain reaction
TE-SS	Tris-EDTA salmon sperm
Th	T helper
TIR	Toll-interleukin-1 receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor alpha receptor
TPA	Phorbol ester
TRIF	Tir-domain-containing adaptor protein inducing IFNβ
Tris	Tris(-hydroxymethyl-) aminomethane
Tyk-2	Tyrosine kinase 2

UPL	Universal ProbeLibrary
UV	Ultraviolet

4. Introduction

4.1 Human papillomaviruses (HPVs)

Human papillomaviruses (HPVs) are small double-stranded DNA viruses, which are classified into cutaneous and mucosal types according to their target tissue. The cutaneous types infect the squamous epithelia of skin, while the mucosal ones infect mucosal epithelia in the genital tract and oropharynx causing a variety of clinical outcomes and most notably cervical cancer (Zur Hausen, 1999).

Persistent high-risk HPV infection is the major aetiological factor for the development of cervical cancer, since over 99% of the cervical cancer biopsy specimens are found to be positive for HPV DNA (Zur Hausen, 1990; Melbye et al, 2002; Zur Hausen, 1996; Walboomers et al, 1999).

Until now, there are more than 100 types of HPVs, but one-third of them target specifically the genital tract epithelium transmitted through sexual contact (Kjaer et al, 2001; Zur Hausen, 2002). The genital types of HPV are categorized into two groups according to their oncogenic potential: high-risk HPVs, which can induce malignant transformations, and low-risk HPVs, which can cause benign genital warts. The high-risk group includes HPV16, HPV18, HPV33, HPV31, and HPV45, whereas the most frequent types in the low-risk HPVs category are HPV6 and HPV11 (Zur Hausen, 2002).

Infection with HPVs first causes low-grade lesions called cervical intraepithelial neoplasia grade 1 (CIN 1), which are mostly eliminated in 1-2 years. In a minority of patients the viruses can persist for decades evading from the immune system. In cases of oncogenic HPV, the low-grade intraepithelial neoplasia (CIN 1) can consequently evolve to high-grade intraepithelial neoplasia and finally to cervical cancer (Ho GY et al, 1998; Jenson et al, 1991;Höpfl et al, 2000; Zur Hausen, 1996). Cervical cancer is the 3-4 most prevalent cancer in women worldwide, where 470,000 new cases are annually recorded (Jemal et al, 2011).

What is more, HPVs can also cause other cancers, where it has been found that close to 93% of anal cancers in USA and up to 25-30% of oropharyngeal carcinoma are associated with human papillomaviruses (Joseph et al, 2008; Zur Hausen, 2000; Herrero et al, 2003). Moreover, these viruses have been identified in penile, vulvar, esophageal and skin carcinoma (Melbye et al, 2002; Zur Hausen, 1996; Zur Hausen, 1990; Wieland et al, 2000; Syrjänen, 2002).

HPVs are not the only risk factor for emergence of cancer, but a number of cofactors are probably additionally involved in carcinogenesis such as smoking, co-infection with human immunodeficiency virus, and immunosuppression (Lie AK, 2000; Kjellberg et al, 2000; Daling et al, 1996).

4.1.1 The HPV proteins

Genomes of mucosal HPVs contain 8 open-reading frames (ORFs), and the products of these genomes are classified into early and late proteins. The early proteins are expressed in the early phase of the virus life cycle in undifferentiated keratinocytes, and these proteins are E1, E2, E1^E4, E5, E6, and E7. On the other hand, the late proteins L1 and L2 are structural proteins, which are subsequently expressed in differentiated epithelial cells forming the icosahedral capsid of the virus (Longworth & Laimins, 2004).

Upon viral infection the first viral genes to be expressed are E1 and E2 genomes, and their products E1 and E2 proteins initiate cooperatively the replication of DNA virus by recruiting the DNA-polymerase and accessory protein of the host cell (Conger et al, 1999).

E1^E4 protein is thought to be able to induce collapse of the keratin network in keratinocytes and may play a role in initiating the differentiation-dependant phase (Doorbar et al, 1991).

E5 protein is expressed early in the productive phase of the viral life cycle (Fehrmann et al, 2003; Genther et al, 2003).

High-risk HPVE6 is an oncoprotein, which has significant effects on the infected epithelial cells. It can cause immortalization and malignant transformation due to its binding to p53, its degradation and/or blocking p53 function. (Huibregste & Beaudenon, 1996; Huibregste et al, 1991; Scheffner et al, 1990). p53 is a tumor suppressor protein, which is activated upon DNA damage inducing cell cycle arrest and apoptosis (Lechner & Laimins, 1994; Ko & Prives, 1996). Furthermore, E6 can indirectly reduce p53 activity by forming a complex with its co-activator p300/CBP. p300/CBP is a histone acetyltranferase which activates p53 by acetylation. As a result of this complex formation the acetylation of p53 is suppressed and finally p53 activity is downregulated (Zimmermann et al, 1999; Patel et al; 1999).

It is important to notice that E6 oncoprotein is alone insufficient for efficient immortalization and malignant transformation in infected cells, but it needs the cooperation of E7 oncoprotein (Hawley-Nelson et al, 1989; Münger et al 1989a). E7 interacts with members of the retinoblastoma (Rb) family of tumor suppressor proteins. p107, p130 and Rb regulate cell cycle progression. The association of E7 with Rb family abrogates function which is essential for the productive stage of the viral life cycle (Münger et al, 1989b; Dyson et al, 1989; Classon & Dyson, 2001; Berezutskaya et al, 1997; Münger et al, 1992) (Fig. 1).

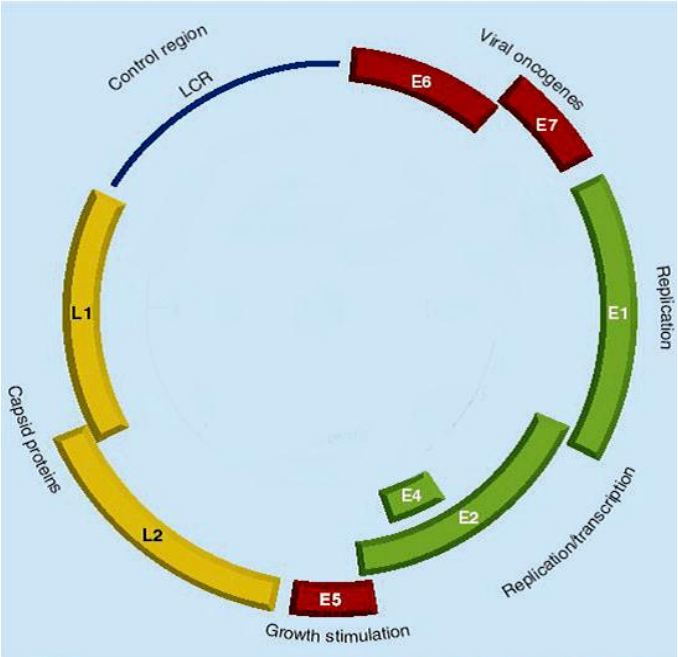

Figure 1 Organization of HPV genome. The early region expresses the early proteins including E1, E2, E4, E5, E6, and E7. The late region of the genome encodes capsid proteins comprising L1 and L2. LCR is a control region. (Adapted from Lie & Kristensen 2008).

4.1.2 HPV life cycle

HPVs are non-enveloped viruses that infect keratinocytes of the stratified epithelium and replicate in their nuclei. Papillomaviruses can enter basal keratinocytes via a microtrauma in the epithelium. Upon the viral entrance into the basal cell, the virus is established in the host nucleus as an extrachromosomal episome replicating concurrently with the host cell in average of 20-100 copies of viral DNA per cell. The virus does not encode a DNA polymerase and other replication factors, so that it has to use those of the host cell (Longworth & Laimins 2004; Fermann & Laimins, 2003). The recruitment of the cellular replication machinery for the virus replication is due to the function of both E1 and E2 viral proteins, which are the first viral proteins to be expressed (Conger et al, 1999).

The productive life cycle of these viruses is strongly restricted to the differentiated keratinocytes (Longworth & Laimins, 2004). The expression of the late viral proteins is limited to the differentiated cells (Laimins LA, 1993). In

normal keratinocytes a basal cell divides into two daughter cells, one of them remains in the basal layer and serves as a reservoir for later cellular divisions, and the other migrates towards the suprabasal layers undergoing differentiation. For differentiation, keratinocytes have to leave the cell cycle. In contrast, in keratinocytes infected with high-risk HPVs the viral oncoproteins can induce re-entry into the cell cycle even in differentiating layers.

The oncoproteins E6 and E7 interfere with the host cell proteins, p53 and (Rb) family members, respectively, and thereby promote vegetative viral replication in the suprabasal strata of the epithelium to thousands of copies per cell (Cheng et al, 1995; Flores et al, 2000). In the upper layers, the late proteins L1 and L2 of the virus are produced and the mature infectious virions are assembled. Mature viruses are released by shedding of the more differentiated epithelial cells (Fig. 2) (Longworth & Laimins 2004).

It has been demonstrated that the viral genome exists as extrachromosomal episome in low-grade intraepithelial neoplasia, while it is found often integrated into the genome of the host cell in the cancerous specimens. This integration of the viral gene into the host keratinocyte sequences takes place within the E2 ORF. Therefore the suppressive effect of E2 protein is lost leading to higher expression of E6 and E7 proteins (Jeon et al, 1995; Choo et al, 1987; Tonon et al, 2001).

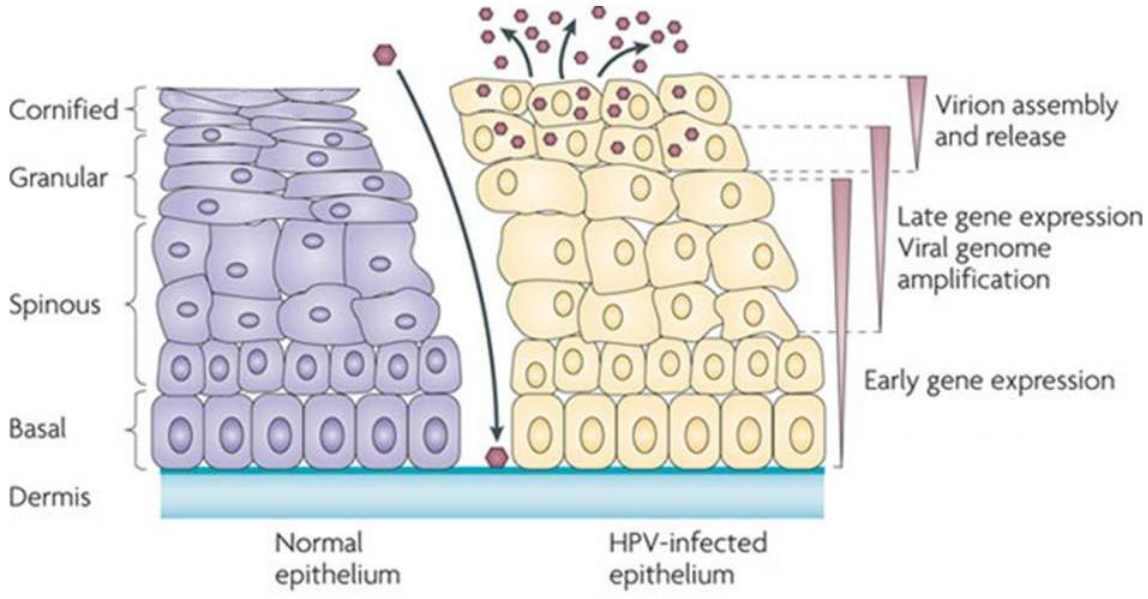

Figure 2 Diagram showing normal epithelium (left) and human papillomavirus (HPV) infected epithelium (right). As the HPV infected cell of the basal membrane replicates, the DNA virus also replicates with it as extrachromosomal DNA. In the upper layers the late genes are expressed and the viral DNA is encased, after that the virions are released into the environment with the discarded epithelial cells. (Modified from Moody & Laimins, 2010).

4.2 The role of interferon in the immune system

Interferon (IFN) was identified as an agent produced from influenza virus infected-cells and this agent was able to protect other cells from viral infection. IFNs are a family of cytokines which possess various biological functions such as a characteristic antiviral activity, regulation of cell growth, and immunomodulatory activity (Pestka et al, 1987; Isaacs & Lindenmann, 1957; Lindenmann, 1982; Tanaka et al, 1998). IFNs have a decisive role in the host defense against viral infection either in autocrine manner, or in paracrine manner by establishment of antiviral state in uninfected cells (Tanaka et al, 1998). IFNs can interfere with the replication and transcription of many viruses (Vilcek, 2006). Interferons can be grouped into three types, type I IFNs, type II IFNs and type III IFNs. Type I IFNs are known as viral IFNs according to their critical role in the early host defense against viral infection, and this type of IFNs comprise IFNα, IFNβ, IFNω, IFNε and IFNκ, while IFNγ is the only member in type II IFNs. There is only one IFNγ

gene and also one IFNβ gene, but there are 13 IFNα genes. The last identified interferons IFNλ1, IFNλ2, and IFNλ3 are classified as type III IFNs, and it has been found that these new interferons are produced in virally infected cells (Takaoka & Yanai, 2006). IFNα and IFNβ, the best characterized type I IFNs, can be produced from several types of cells in response to viral and other microbial infections, while only special immune cells including natural killer (NK) cells, $CD4^+$ T helper 1 (T_h1) cells and $CD8^+$ cytotoxic T cells are capable of IFNγ excretion. IFNs demonstrate their pleiotropic properties by binding to specific cell surface receptors and this binding activates the expression of more than 30 different cellular proteins through the Janus kinase/signal transducers and activators of transcription JAK-STAT signaling pathway, whose components belong to two types of transcription factors. The first is termed as Janus kinase family (JAK) and comprises Jak-1, Jak-2 and Tyk-2 kinases, whereas the second is the signal transducer and activator of transcription family (STAT). Both, Stat1 and Stat2, are essential player in the IFN-signaling pathways. Upon the treatment of the cell with IFN α/β, Jak-1 and Tyk-2 kinase activate Stat1 which in turn forms heterodimer with Stat2, and then the Stat1-Stat2 heterodimers translocate to the nucleus and together with p48 form interferon stimulated gene factor 3 (ISGF3), complex causing the induction of IFN-inducible genes. On the other hand, when the cell is exposed to IFNγ, Jak-1 and Jak-2 are activated and consequently phosphorylate Stat1, which undergoes homodimerization and then nuclear translocation leading to induction of gene expression (Fig. 3) (Pestka et al, 1997; Silvennoinen et al, 1993; Katze et al; 2002; Velazquez et al, 1992; Darnell et al, 1994).

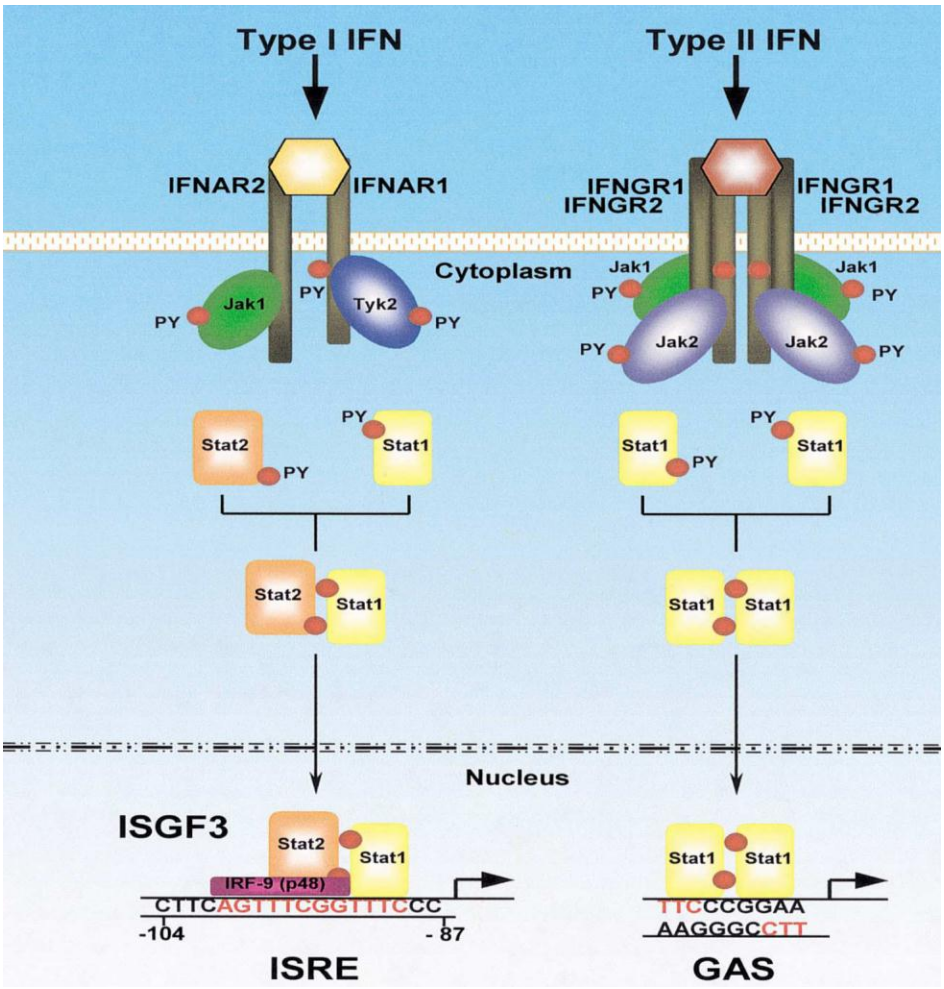

Figure 3 Diagram showing the induction of Jak-Stat pathway by IFNs. IFNα/β exhibit their actions by binding to their specific receptors which leads to activation of Jak1 and Tyk2 of the janus kinase family (Jak) resulting in phosphorylation of Stat1 and Stat2. Stat1 and Stat2 heterodimer, then translocate into the nucleus and bind to protein 48 (IRF9) forming the ISGF3 complex on the interferon response element (ISRE). On the other hand, the association of IFNγ with its receptor activates Jak1 and Jak2, which in turn activates Stat1. Activated Stat1 undergoes homodimerization and then nuclear translocation which leads to binding on IFNγ-activated sequence (GAS). (Koromilas et al, 2001).

4.3 The IRF family

Interferon regulatory factors (IRFs) are group of nine transcription factor proteins (IRF-1 to IRF-9) inducible by IFNs. They exhibit diverse biological functions comprising apoptosis, immune response, cell growth regulation, oncogenesis and host defense (Romieu-Mourez et al, 2006; Sato et al, 2000; Pitha et al, 1998;

Honda et al, 2005; Nakamura et al, 2001; Nguyen et al, 1997). It has been elucidated that the two members IRF3 and IRF7 of this family are essential regulators of the induction of IFNα/β as an innate immune response to viral infection (Sato et al, 2000; Wathelet et al, 1998). Interferon regulatory factor 3 (IRF3) is constitutively expressed in the cytosol of cells in a latent form, but viral infection triggers the phosphorylation of IRF3, dimerzation and nuclear translocation. This in turn provokes IFNα4 and IFNβ production. IRF3 has a stronger stimulatory impact on IFNβ genes than on IFNα genes (Juang et al, 1998; Yoneyama et al, 1998).

Interferon regulatory factor 7 (IRF7) is mapped in human to chromosome 11, and it has short half-life (only 0.5-1 h). The first detection of IRF7 was in the context of research on Epstein-Barr virus (EBV) (Zhang & Pagano, 1997; Zhang & Pagano, 2002).

IRF7 is expressed in the spleen, thymus and peripheral blood lymphocytes (PBL), but it is also found at low levels in all tissues (Zhang & Pagano, 1997; Au et al, 1998). Many factors can activate the expression of IRF7 such as IFNα, IFNβ, viral infection, lipopolysaccaride (LPS), DNA-damaging agents like ultra-violate light (UV), in addition to chemical substances like sodium butyrate and phorbol ester (i.e. TPA) (Sato et al, 2000; Lin et al, 2000; Zhang & Pagano; 2002).

Both IRF3 and IRF7 are key regulators of IFN-mediated responses to viral infection. Two distinct phases in interferon induction can be distinguished achieving a robust innate immune defense against viral infection (Au et al, 1998; Honda et al, 2005; Sato et al; 1998; Kumar & Korutla 1995; Kimura et al 1996; Marie et al, 1998).

4.4 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) are type I transmembrane proteins that have key roles in the innate and also adaptive immune response. They recognize pathogen-associated molecular patterns (PAMPs), which may be lipids, lipoproteins, proteins and nucleic acids drawn from a large spectrum of microbes including viruses (Akira et al, 2001; Akira et al, 2006). Up to now, they are grouped into two subgroups according to their cellular localization and related PAMP. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 belong to the first group, which exists on the cell surface and recognizes membrane parts of the microbes such as lipids, proteins and lipoproteins. Moreover, the second group includes TLR3, TLR7, TLR8 and TLR9 which locate within the cells and recognize nucleic acids (Kawai & Akira, 2010). Each TLR consists of leucine-rich domain, that binds to the pathogen, transmembrane domain, and intracellular toll-interleukin 1 (IL1) receptor (TIR)-domain, whose role is critical in the activation of signal transduction following either myeloid differentiation factor 88 (MyD88)-dependent pathway or TIR-domain-containing adapter-inducing interferon beta (TRIF)-dependent pathway activation. As a result, the activation of both pathways leads to production of inflammatory cytokines and type I interferon through the activation of nuclear transcription factor κB (NF-κB) and interferon regulatory factors IRF7 or IRF3. These TLR signaling serves as an antiviral way of the host cell to fight against viral infection (Gay & Gangloff, 2007; Jin & Lee, 2008; Akira et al, 2006; Kawai & Akira, 2009). Furthermore, both figure (4) and figure (5) respectively, show how cell surface TLRs and intracellular TLRs recognize the PAMPs that finally cause the production of an inflammatory cytokines and type I IFN by recruiting MyD88 or TRIF.

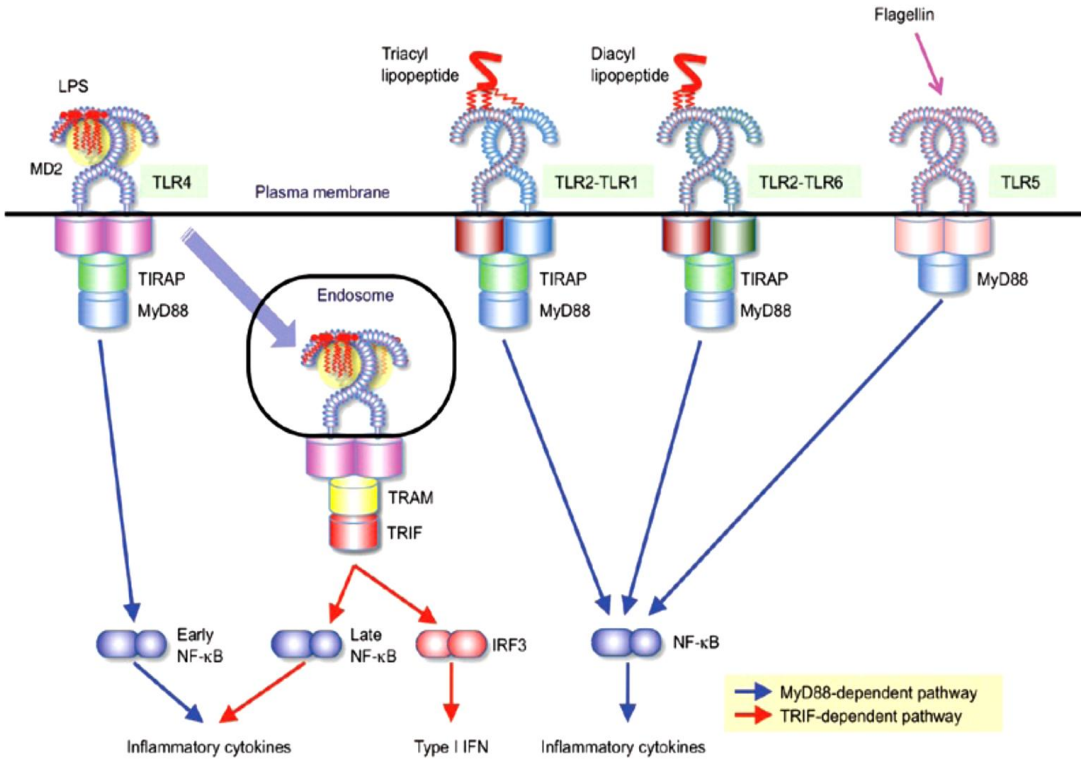

Figure 4 PAMP recognition by cell surface TLRs. TLR4 recognizes LPS inducing signal transduction through either MyD88-dependent pathway, or TRIF-dependent pathway, which leads consequently to induction of type I IFN and inflammatory cytokines. TLR2-TLR1, TLR2- TLR6 heterodimers and TLR5 trigger induction of inflammatory cytokines by recruiting the MyD88-dependent pathway (Kawai & Akira, 2010).

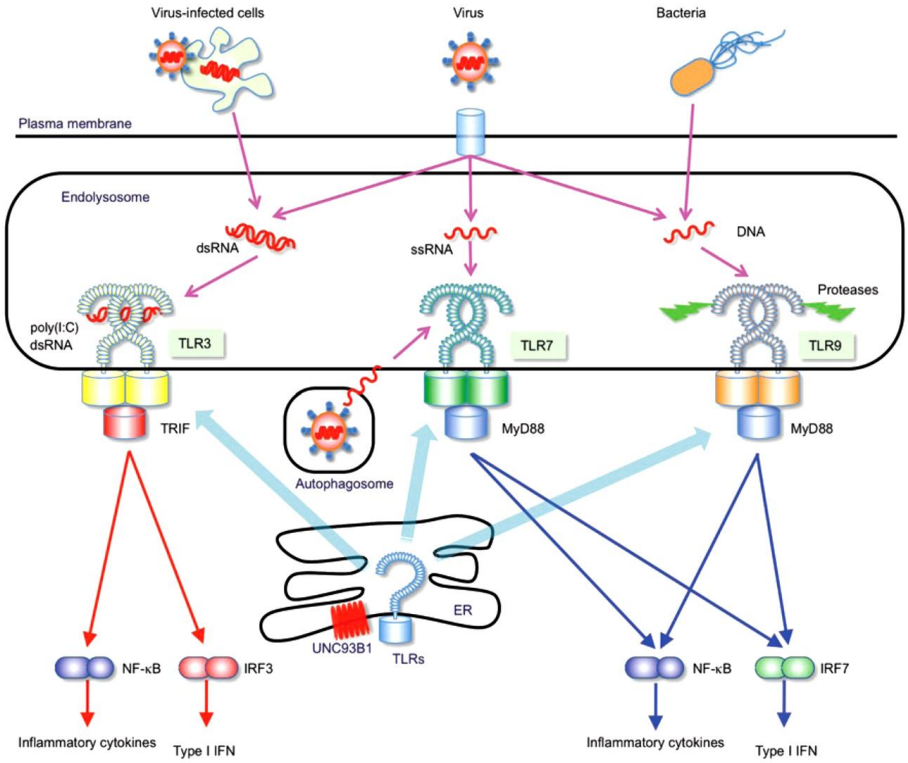

Figure 5 PAMP recognition by intracellular TLRs. TLR3 recognizes dsRNA derived from viruses and then triggers the induction of type I IFN and inflammatory cytokines through TRIF-dependent pathway. TLR7 recognizes ssRNA activating the production of type I IFN and inflammatory cytokines through the MyD88-dependent pathway. TLR9 recognizes DNA extracted from viruses, and then activates the MyD88-dependent pathway resulting in production of inflammatory cytokines and type I IFN by triggering of NFκB and IRF7, respectively (Kawai & Akira, 2010).

4.5 Tumor necrosis factor alpha TNFα

Tumor necrosis factor alpha TNFα is a proinflammatory cytokine produced upon infection by a wide range of cells such as macrophages, $CD4^+$ T-lymphocytes, $CD8^+$ T-lymphocytes, B-lymphocytes, NK cells and neutrophils. Inflammation, induction of immune responses and host defense belong to the various functions of TNFα, which achieves these functions by binding specifically to TNF receptors, which belong to the TNF receptor superfamily (Mak & Yeh, 2002; Idriss & Naismith, 2000). As a consequence of the binding of TNFα to its receptors many

signaling pathways are activated particularly the NF-κB pathway inducing a number of transcription factors such as IRF7 (Lu et al, 2002).

4.6 Human papillomavirus (HPV) and interferon (IFN)

Human papillomavirus proteins interfere with the cellular response to IFNs by multiple mechanisms. The E6 oncoprotein targets many components of the IFN-signaling pathway, where it is known now that E6 binds to Tyk2 kinase blocking the induction of the Jak-Stat pathway by interferon (Li et al, 1999). Moreover, it has been reported that E6 protein interacts with IRF3 (Ronco et al, 1998; Zimmermann et al, 1999; Patel et al; 1999). In addition to E6 oncoprotein, E7 oncoprotein can also mediate the IFN-signaling pathway either by binding to IRF1 and blocking its function (Park et al, 2000; Perea et al, 2000), or by binding to p48 which is considered a pivotal part of the ISGF3 complex causing suppression of the IFN-inducible genes. It has been found that the response to the IFN-treatment is correlated with the levels of E7 oncoprotein (Barnard & McMillan, 1999). Furthermore, Chang and Laimins (2000) have reported that the HPV31 proteins target Stat1 causing the disruption of its decisive role in mediating the response to IFN, and then resulting in repression of IFN-inducible genes. The previous way is utilized by HPV in modulating the IFN-signal pathway and may serve as a strategy to evade from the immune defense and persist.

4.7 IFN therapy

IFN have been variously used for therapy of HPV-induced diseases, however with mixed results. Genital warts caused by low-risk types of HPV have been eliminated after the IFN application in some studies. In contrast, the responses of lesions triggered by high-risk types of HPV have varied even to a higher degree. More important for IFN-responsiveness was the integration status of the viral DNA in the nucleus of the host cell. Whereas lesions harboring episomal viral DNA have been cleared, more efficient lesions with integrated viral DNA have been more resistant to the treatment with IFN (Beglin et al, 2009; Koromilas et al, 2001). Terenzi and others (2008) have found that the interferon-inducible protein 56 (p56) plays the main role in the response to IFN-treatment by binding to the

viral protein E1 which is a key factor in the viral DNA replication. The interaction of p56 with the E1 protein leads to sequestering of the E1 protein from the nucleus to the cytoplasm resulting in inactivation. Accordingly the viral DNA replication is inhibited.

4.8 Interferon-induced GTP-binding protein Mx1

Mx1 gene is one of the interferon-stimulated genes (ISGs) firstly characterized in mice as an antiviral factor against influenza virus infection (Aebi et al, 1989; Haller et al, 1998; Horisberger et al, 1983; Lindenmann, 1962). Haller and Kochs (2002) have demonstrated that Mx1 can repress the replication of certain RNA viruses. Mx1 protein exists normally in the cytoplasm of the cell in undetectable levels, but after exposure to type I IFN, Mx1 production is stimulated reaching up to 1% of the cytosolic proteins, whereas weak activation of Mx1 gene expression has been noticed by IFNγ, and type III IFN (Holzinger et al, 2007; Aebi et al, 1989; Horisberger, 1992). On the other hand, while IFNα and IFNβ are considered as potent inducers of Mx1, Simon and others (1991) have demonstrated that Mx1 gene expression does not respond to the stimulation with tumor necrosis factor alpha (TNFα).

As a result of the previous considerations, Mx1 gene expression is commonly used as a unique marker for the detection of the various biological actions of IFN in the context of the IFN-therapy or during viral infection. Hence Mx1 can serve as a positive control to the induction by interferon.

4.9 CC chemokine ligand 20 (CCL20)

CCL20 is a chemokine known previously as macrophage inflammatory human protein-3 alpha (MIP-3α) or liver and activation-regulated chemokine (LARC). CCL20 is constitutively expressed at low levels in the cell and inducible by poly I:C (dsRNA) and many stimuli like lipopolysaccharides (LPS). Sperling and others (2012) have demonstrated novel CCL20 induction pathway by CCAAT-enhancer-binding proteins C/EBPβ in normal human keratinocytes. IL-1β and TNFα have been established as strong inducers of the CCL20 induction through activation of the NF-κB signal pathway, while IFNγ has no effect on CCL20

production (Reibman et al, 2002; Nakayama et al, 2001; Fujiie et al, 2001; Hosokawa et al, 2005; Schutyse et al, 2000; Tohyama et al, 2001). Therefore, CCL20 can be used in gene expression studies as a reliable marker for the TNFα bioactivities.

5. Aim of the study

More than 99% of cervical carcinomas are positive for HPV DNA. The genital high-risk types HPV16 and HPV18 are account for more than 70% of cervical cancer. Interferons represent an important part of the host defense against viral infection. In HPV-mediated skin carcinogenesis, IRF7 binds to viral DNA and increases the activity of the late promoter of a cutaneous high-risk HPV8 however, the role of IRF7 in genital carcinogenesis is so far unknown.

In this study we examined the expression of the transcription factor IRF7 after TNFα or IFNβ stimulation. We focused on the differences between normal HPV-negative keratinocytes in comparison to HPV16- or HPV18-infected non-malignant as well as malignant cells.

6. Material and Methods

6.1 Material

6.1.1 Eukaryotic cell lines

C4-I: HPV18-positive human cervical carcinoma cell line.

CaSki: HPV16-positive human cervical carcinoma cell line.

HeLa: HPV18-positive human adenocarcinoma cell line.

HPK1A: human foreskin keratinocytes immortalized with HPV16. Contains 2-3 integrated HPV16 genomes.

K51: human foreskin keratinocytes immortalized with HPV18.

SW756: HPV18-positive cells of a differentiated cervical squamous cell carcinoma.

EXLNp5: primary exocervical normal human keratinocytes (HPV negative) in passage 5.

NFK13p3: primary normal human foreskin keratinocytes (HPV negative) in passage 3.

6.1.2 Culture media and reagents for cell culture

Media and additives

Keratinocyte Basal medium	Lonza, Köln
KBM-Gold medium	
Trypsin/EDTA, 0.25mg/ml	Lonza, Köln
Dulbecco's modified eagle medium (DMEM)	PAA, Pasching
Fetal calf serum (FCS)	PAA, Pasching

Penicillin, Streptomycin	PAA, Pasching
Penicillin, 10.000 Units/ml	
Streptomycin, 10 mg/ml	
Sodium-pyruvate, 11g/l	PAA, Pasching
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, Steinheim
PBS	PAA, Pasching

Complete medium for NFK and EXLN cells

KBM-Gold medium with KGM-Gold Single Quots

Complete medium for the other cells

DMEM with 10% FCS, 1% sodium-pyruvate and 1% Penicillin/Streptomycin

Freezing medium

90% FCS with 10% DMSO

6.1.3 Reagent system

6.1.3.1 RNA isolation

QIAschredder	QIAGEN, Hilden
RNeasy kit	QIAGEN, Hilden
RNase-free DNAse set	QIAGEN, Hilden

6.1.3.2 cDNA synthesis and RT-PCR

The super-script II system from Invitrogen Corporation, Karlsruhe, was used for cDNA synthesis. In addition, for the quantitative Real-Time PCR a kit FastStart Taq DNA polymerase, dNTPack 5U/µl from company of Roche, Mannheim was used.

This kit included:

FastStart Taq DNA polymerase, 5U/µl

PCR buffer, 10 x concentrated without $MgCl_2$

$MgCl_2$, 25 mM

Nucleotid mix (dATP, dGTP, dCTP, dTTP)

For the quantification of cDNA special fluorescent probes from the human Universal ProbeLibrary (UPL) from Roche Company, Mannheim were used.

6.1.4 Cytokines for stimulation

Betaferon IFNβ-1b 250 µg/ml	Schering
TNFα 10^6 U/ml	Boehringer

6.1.5 Chemicals

Agarose	Lonza, Köln
β-Mercaptoethanol	Sigma-Aldrich, Steinheim
Bovine Serum Albumin (BSA)	Fermentas, St. Leon-Roth
Chloroform	Roth, Karlsruhe
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, Steinheim
Ethanol	Roth, Karlsruhe

Ethylenediaminetetraacetic acid (EDTA)	PAA, Pasching
Ethidiumbromide	Sigma-Aldrich, Steinheim
Formaldehyde	Roth, Karlsruhe
Isopropanol	Roth, Karlsruhe
Nucleotide (dNTPs)	Invitrogen, Karlsruhe
Salmon sperm DNA	Invitrogen, Karlsruhe
Tris-EDTA buffer solution (TE-buffer)	BioUltra Fluka Analytical
Trizol reagent	Invitrogen, Karlsruhe
Tween 20	Serva, Heidelberg

6.1.6 Oligonucleotide for real time PCR

The real time PCRs were designed with the Universal ProbeLibrary (UPL) Program of Roche Company, Mannheim. The oligonucleotides were purchased from the company Sigma-Aldrich, Steinheim.

Table 1 Primers, $MgCl_2$ concentration and probe number used for RT-PCR of GAPDH.

GAPDH	Sense and antisense primer	5'-CTGACTTCAACAGCGACACC-3' 5'-TGCTCTAGCCAAATTCGTTGT-3'
	$MgCl_2$ Concentration	2mM
	No. of UPL probe	25

Table 2 Primers, $MgCl_2$ concentration and probe number used for RT-PCR of IRF7.

IRF7	Sense and antisense primer	5'-TCGACTTCAGAGTCTTCTTCCA-3' 5'-CGAAGCCCAGGTAGATGGTA-3'
	$MgCl_2$ Concentration	4mM
	No. of UPL probe	31

Table 3 Primers, $MgCl_2$ concentration and probe number used for RT-PCR of Mx1.

Mx1	Sense and antisense primer	5'-CTGACCTTGCCTCTCCACTT-3' 5'-ACTGTCAGGAGTTGCCCTTC-3'
	$MgCl_2$ Concentration	5mM
	No. of UPL probe	34

Table 4 Primers, $MgCl_2$ concentration and probe number used for RT-PCR of CCL20.

CCL20	Sense and antisense primer	5'-GCAGTCAAAGTTGCTTGCTTC-3' 5'-GCTGCTTTGATGTCAGTGCT-3'
	$MgCl_2$ Concentration	4mM
	No. of UPL probe	39

6.1.7 Plastic ware

Plastic ware was used from the company of Greiner, Frickenhausen; Nunc, Wiesbaden; Sarstedt, Nümbrecht; TPP, Trasadingen, Schweiz and VWR, Darmstadt.

6.1.8 Equipments

Centrifuges	Heraeus Megafuge 1,0 R
	Heraeus Picofuge
	Sigma-Aldrich 202 MK
UV-Spectrophotometer	NanoDrop model 2000C, Thermo scientific
Microscope	Leica DMI600 B
Neubauer counting chamber	NeoLab, Heidelberg
Real-Time PCR Cycler	LightCycler 480, company Roche Mannheim
UV-Gel documentation	BioRad ChemiDoc XRS^{+}

6.1.9 Computer programs and internet websites

Design of primer	PrimerBlast of NC (www.ncbi.nlm.nih.gov/tools/primer-blast/)
Design of primers for qRT-PCR	Universal ProbeLibrary of the company Roche,Mannheim
	(www.rocheappliedscience.com
	/sis/rtpcr/upl/ezhome.html)
Establishment of the diagrams and statistics	Microsoft Excel 2007
Literature	Pubmed, hompage of NCBI (www.ncbi.nlm.nih.gov/pubmed)
Establishment of the text	Microsoft Word 2007

6.2 Methods

6.2.1 Cell culture

6.2.1.1 Cultivation of the cells

The cells were cultivated in DMEM complete medium at 37°C with 5% CO_2. To harvest the cells, medium was discarded, the cells were washed with 2ml PBS, 4ml Trypsin/EDTA was added and the cells were incubated for 5 minutes at 37°C. The detaching of the cells was controlled by using a microscope (Leica DMI600 B).

The enzymatic activity of Trypsin was stopped by adding 6ml DMEM complete medium. The cells were seeded in the desired ratio according to the cell density and the growth rate.

6.2.1.2 Cell number determination

To calculate the cell number, the medium was discarded and the cells were washed with 2ml Trypsin/EDTA and then incubated with 4ml Trypsin/EDTA for 5 minutes at 5% CO_2. The detaching of the cells was checked up by microscope. 6ml DMEM complete medium was added to stop the activity of Trypsin and then centrifuged for 5 minutes at 1200 rpm at room temperature. The supernatant was removed and the pellet was resuspended in 20ml DMEM complete medium.

10µl Trypan blue was added to 10µl of the cell suspension in 1.5ml reaction tube. The cells were incubated for 2 minutes at room temperature and then 10µl were calculated in the Neubauer-chamber.

By using microscope, the cells were counted in 4 large squares. The mean was calculated and the required number of the cells was calculated as the following:

$$\text{The required number} = \text{the calculated mean} \times 2 \times 10^4 \text{ cells/ml}$$

(2 means the ratio of dilution)

6.2.1.3 Seeding of the cells

1.5×10^6 cells were seeded in $6cm^2$ cell culture dishes, and finally the volume was completed to 3ml with DMEM complete medium. The cells were incubated overnight in 5% CO_2 at 37°C.

6.2.1.4 Seeding of the primary cells (NFK and EXLN)

250 000 cells were seeded in 6 well cell culture dishes and then the volume was completed to 2ml with KBM-Gold medium with additives (Hydrocortisone 0.5 ml, Transferrin 0.5 ml, Epinephrine 0.25 ml, GA-1000 0.5ml, BPE 2.0 ml, rhEGF 0.5 ml and insulin 0.5 ml). Trypsin/EDTA was used for detaching the cells, TNS for stopping this detaching and HEPES for washing the cells. The cells were incubated overnight in 5% CO_2 at 37°C.

6.2.1.5 Thawing of the cells

The cryo-tubes with the cells were taken from -80°C storage and quickly thawed at 37°C. The cells were centrifuged in 4ml medium at 1200 rpm for 10 minutes at room temperature. The supernatant was discarded and the cells were resuspended in 4ml medium and transferred in 75 cm^2 cell culture flask with 20ml medium.

6.2.1.6 Freezing of the cells

The cultivated cells were resuspended with 4ml freezing medium consisting of 10% DMSO and 90% FCS. The cell suspension was aliquoted in cryo-tubes that are already on ice. The cryo-tubes were left at -80°C for few days, and then stored with liquid nitrogen.

6.2.2 Stimulation of the cells

Tumor necrosis factor alpha (TNFα), 1×10^6 U/ml, was diluted to 1×10^3 U/ml in DMEM medium without additives.

Interferon beta (IFNβ), 8×10^6 U/ml, was diluted to 1×10^2 U/ml in DMEM medium without additives.

From the cells, which were incubated overnight, the medium was removed. Then 2ml from the diluted TNFα and 2ml IFNβ were added to each dish. The dishes were incubated for 16 hours in 5% CO_2 at 37°C.

6.2.3 RNA-Isolation

a) RNeasy Kit (Qiagen)

Medium was completely removed from the culture dishes and the cells were washed with PBS.

The cells attached to the bottom of the dishes were disrupted by adding 600µl RLT buffer and scraping with cell scraper. Lysate was directly pipetted into a Qiashredder spin columns placed on 2ml collection tubes and then centrifuged for 2 minutes at 11000 rpm by using Heraeus Picofuge centrifuge. Flow was mixed well with 600µl of 70% Ethanol. The mixture was applied including any precipitate that may have formed, to RNeasy mini columns placed on 2ml collection tubes and centrifuged for 15 seconds at 11000 rpm, so that the RNA bound to the membrane. Then the spin column membrane was washed with 350µl RW1 buffer. Next, a 10µl DNase in 70µl RDD buffer was added to each column and incubated for 15 minutes at room temperature in order to remove the rests of DNA from the RNA. Washing was done three times: the first one was done with 350µl RW1 buffer, second with 500µl RPE buffer, and the last one with 500µl RPE buffer. For elution, the RNeasy columns were transferred to a new 1.5ml collection tube and then 50µl RNase free water was pipetted directly on the RNeasy silica gel membrane and afterwards centrifuged for 1 minute at 11000 rpm. The concentration of the RNA was measured by NanoDrop model 2000C, Thermo scientific.

b) Trizol/Phenol-Chloroform method (Invitrogen, Karlsruhe)

The medium was discarded from the culture dishes and the cells were washed with PBS. 1ml Trizol was added and incubated for 5 minutes at room temperature then lysed by repeated pipetting. Lysate was flowed in 2ml reaction tube. 200µl Chloroform was added and the tubes were shaked strongly for 15

seconds by hands and incubated for 2-3 minutes at room temperature. The tubes were centrifuged for 15 minutes at 4ºC at 12000 rpm. The squamous phase was transferred to fresh tubes, 500µl Isopropyl was added and incubated for 10 minutes at room temperature, then centrifuged for 10 minutes at 4ºC and 12000 rpm. As a result, the RNA formed gel like pellet on sides and bottom of the tubes. The supernatant was removed and the pellet was washed with 1000µl of 70% Ethanol, then centrifuged, shortly dried and then lysed in 51µl EDTA 0.001M pH7, 6µl DNase buffer, 3µl RNase , and 2µl RNasin. This mixture was vortexed, centrifuged and incubated for 20 minutes in water bath at 37ºC, then for 15 minutes in water bath at 70ºC. The concentration of RNA was measured by NanoDrop model 2000C, Thermo scientific.

6.2.4 RNA-gel

After isolating of RNA, an electrophoresis was done on an agarose gel to check the quality of RNA. Ethidium Bromide was used as a dye and UV-light was used for controlling. To prepare 1% gel, 0.5 agarose, 31ml DEPC water and 10ml of 5 × RNA-running buffers were heated in the microwave and then 9ml formaldehyde was added. 1µg RNA was mixed with 5µl loading buffer which contained Ethidium Bromide, and then incubated for 15 minutes in water bath at 65ºC. The samples were loaded on the gel and electrophoresed for 90 minutes at 80 volt. A controlling with UV-light was done to estimate the quality of the RNA.

When two distinct bands 18s and 28s RNA were visible; the RNA was intact (Fig. 6).

A) Intact RNA **B)** Degraded RNA

Figure 6 Verification of isolated RNA on agarose gel. (A) Intact RNA (18s and 28s RNA), (B) degraded RNA.

6.2.5 cDNA synthesis

The enzyme Reverse Transcriptase synthesized the DNA-strand from the RNA-template by using the SuperScript II system of Invitrogen.

The following components were pipetted:

Component	Amount
Random primer 500µM	1 µl
RNA	0.5 µg
d NTPs 10mM	1µl
Sterile water free of RNase	to 12µl

The mixture was heated to 65 ºC for 5 minutes in a water bath and chilled quickly on ice and then the following components were added:

Component	Amount
5 x First-Strand Buffer	4µL
0.1 M DTT	2µL

The contents of the tube were mixed gently and incubated in water bath 42 ºC for 2 minutes. After that, 1µl of Reverse Transcriptase enzyme was added then mixed gently by pipetting up and down. Next, the tubes were incubated in water bath at 42 ºC for 50 minutes and at 70 ºC for 15 minutes to inactivate the enzyme. A dilution 1:10 was done with sterile water free of RNase for RT-PCR.

6.2.6 Real-time PCR (RT-PCR)

Polymerase chain reaction (PCR) is an amplification of a specific DNA sequence by using primers which bind to the complementary sequences in the target DNA.

Consequently, a large number of copies of DNA may be obtained by doing 30-40 cycles of 3 steps:

a) Denaturing of the double-stranded DNA for 1 minute at about 95 ºC by disrupting the hydrogen bonds between the complementary bases.
b) Annealing of the reverse and forward primers to the complementary DNA sequences for 45 seconds at about 50-60 ºC. The primers are only to start the amplification.
c) Extending of the annealed primers by DNA polymerase, which reads from 3' to 5' and adds the nucleotides from 5' to 3', in presence of deoxy nucleoside triphosphates (dNTPs) for 2 minutes at about 70 ºC depending on the DNA polymerase used.

There is an exponential increase of the number of the gene due to the copying both strands during PCR (Fig. 7).

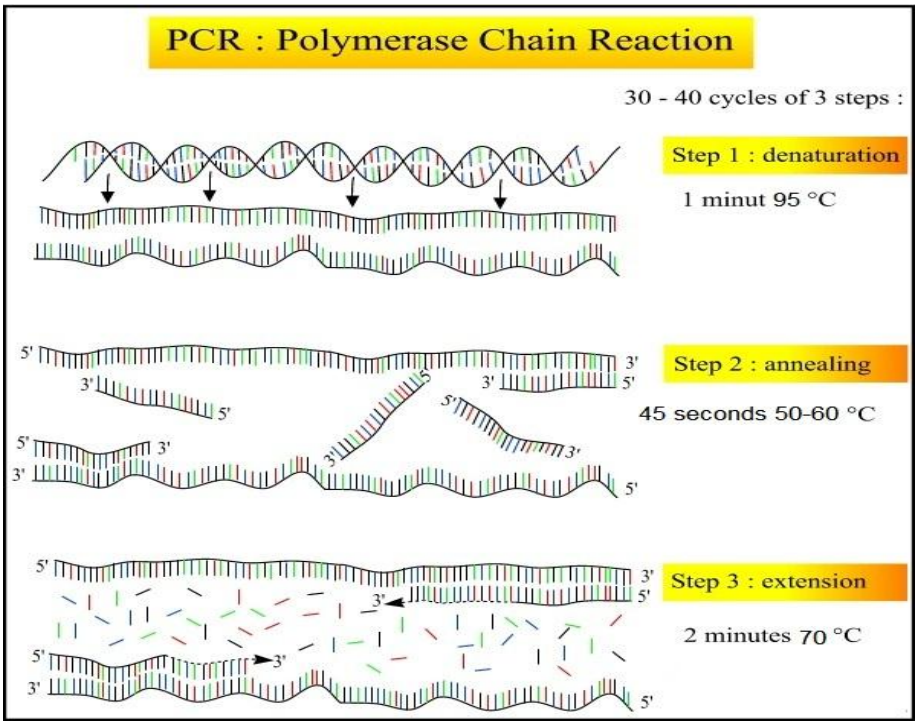

Figure 7 The three steps of PCR principle. The first step is denaturing of the double-stranded DNA for 1 minute at about 95 ºC. Secondly is annealing of the reverse and forward primers to the complementary DNA sequences for 45 seconds at about 50-60 ºC. Thirdly is extending of the annealed primers by DNA polymerase for 2 minutes at about 70 ºC.

Real-time PCR (RT-PCR) or quantitive PCR (qPCR) enables both detection and quantification of one or more specific sequences in the gene at every cycle by using fluorescent dyes which can be specific or non specific dyes.

Non specific dye like SYBR green intercalates with any double-stranded DNA including non specific PCR products such as primer dimer, while a specific dye like TaqMan binds only to the specific PCR product.

TaqMan is an oligonucleotide probe, which consistes of a fluorophore (reporter) at the 5'-end and a quencher at the 3'-end (Fig. 8). As long as the quencher (Q) is close to the reporter (R), it prevents the emission of the fluorescence. When the DNA polymerase extends the primer, the activity of 5'-3' exonuclease of polymerase cleaves the probe inhibiting the activity of the quencher and releasing the fluorescence of the reporter (Fig. 9).

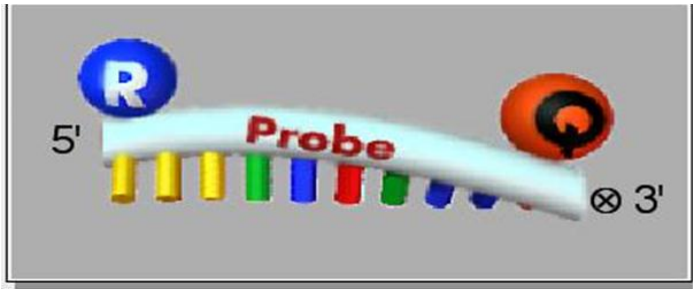

Figure 8 Diagrammed structure of TaqMan showing oligonucleotide with reporter at 5'-end and quencher at 3'-end.

The geometric increase of the fluorescence, which is related to the exponential increase of the product, is detected and measured in RT-PCR at each cycle allowing the determination of the threshold cycle (C_t).

Threshold cycle is the cycle at which the instrument can distinguish the fluorescence emitted by amplification of the gene from the background noise, and it is very important to the accuracy of the resultes.

The efficiency of RT-PCR can be calculated using the slope of the standard curve, which is automaticily made by LightCycler 480 software from readings of the amplification of the plasmid dilution series, according to this equation:

$$E=10^{[-1/slope]}$$

Where:

E: Real-Time PCR efficiency

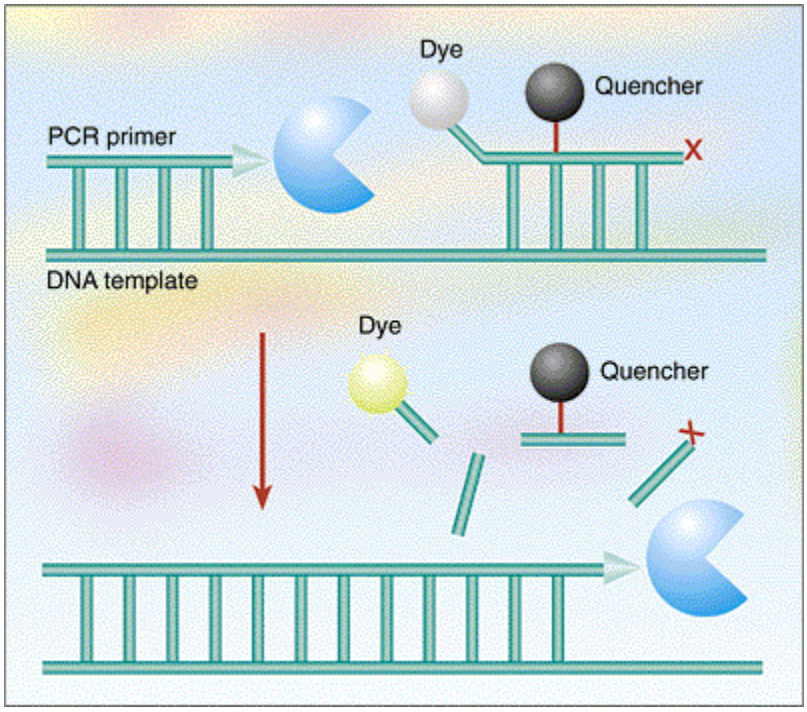

Figure 9 The principle of TaqMan probe work. At the first the reporter fluorescence is quenched, when an extension of the primer by DNA polymerase takes place, the probe is cleaved and a emission of the reporter is detected**.**

To prepare a master mix for RT-PCR the components in tables (5, 6, 7, and 8) were pipetted.

Table 5 The components and their amounts and final concentration applied for RT-PCR of Mx1 gene.

hu Mx1 gene		
Component	**Amount**	**Final concentration**
H_2O	3.6 µl	-
$MgCl_2$ 25 mM	4 µl	5 mM
10 x Taq-buffer	2 µl	1x
TE-SS 40 ng/µl	2 µl	2 ng/µl
DMSO	1 µl	5 %
Tween 20 10 %	1 µl	0.5 %
BSA 20 ng/µl	1 µl	0.5 ng/µl
d'NTPs 10 mM	0.5 µl	200 nmol
Probe Nr°. 34 1 µM	0.4 µl	20 nmol
Primer F Nr°. 153 10 µM	0.4 µl	250 nmol
Primer R Nr°. 154 10 µM	0.5 µl	250 nmol
Taq 5 U/ µl	0.1 µl	0.5 U

Table 6 The components and their amounts and final concentration applied for RT-PCR of IRF7 gene.

hu IRF7 gene		
Component	**Amount**	**Final concentration**
H_2O	4.4 µl	-
$MgCl_2$ 25 mM	3.2 µl	4 mM
10 x Taq-buffer	2 µl	1x
TE-SS 40 ng/µl	1 µl	2 ng/µl
DMSO	1 µl	5 %
Tween 20 10 %	1 µl	0.5 %
BSA 20 ng/µl	0.5 µl	0.5 ng/µl
d'NTPs 10 mM	0.4 µl	200 nmol
Probe Nr°. 31 1 µM	0.4 µl	20 nmol
Primer F Nr°. 163 10 µM	0.5 µl	250 nmol
Primer R Nr°. 164 10 µM	0.5 µl	250 nmol
Taq 5 U/µl	0.1 µl	0.5 U

Table 7 The components and their amounts and final concentration applied for RT-PCR of GAPDH gene.

hu GAPDH gene		
Component	**Amount**	**Final concentration**
H_2O	6 µl	-
$MgCl_2$ 25 mM	1.6 µl	2 mM
10 x Taq-buffer	2 µl	1x
TE-SS 40 ng/µl	1 µl	2 ng/µl
DMSO	1 µl	5 %
Tween 20 10 %	1 µl	0.5 %
BSA 20 ng/µl	0.5 µl	0.5 ng/µl
d'NTPs 10 mM	0.4 µl	200 nmol
Probe Nr°. 25 1 µM	0.4 µl	20 nmol
Primer F Nr°. 23 10 µM	0.5 µl	250 nmol
Primer R Nr°. 24 10 µM	0.5 µl	250 nmol
Taq 5 U/µl	0.1 µl	0.5 U

Table 8 The components and their amounts and final concentration applied for RT-PCR of CCL20 gene.

hu CCL20 gene		
Component	**Amount**	**Final concentration**
H_2O	4.4 µl	-
$MgCl_2$ 25 mM	3.2 µl	4 mM
10 x Taq-buffer	2 µl	1x
TE-SS 40 ng/µl	1 µl	2 ng/µl
DMSO	1 µl	5 %
Tween 20 10 %	1 µl	0.5 %
BSA 20 ng/µl	0.5 µl	0.5 ng/µl
d'NTPs 10 mM	0.4 µl	200 nmol
Probe Nr°. 39 1 µM	0.4 µl	20 nmol
Primer F Nr°. 7 10 µM	0.5 µl	250 nmol
Primer R Nr°. 53 10 µM	0.5 µl	250 nmol
Taq 5 U/µl	0.1 µl	0.5 U

Master mix was distributed to 96 wells PCR plate as 15µl per well. Afterwards, 5µl of the standard was added to the first row and 5µl of the diluted cDNA to the other rows of the plate, where the standard was diluted in TE-Salmon Sperm 5 ng/µl.

Table 9 The studied genes and their corresponded standards.

Studied gene	Standard
	Plasmid $_P$ BSK I+ hu Mx1
hu Mx1	**4948 bp**
	4550bp
	Plasmid $_P$ GEX-5x-2-hu IRF7
hu IRF7	**6400 bq**
	26 ng/µl Nanodrop (miniprep)
	Plasmid $_P$ CMV-SPORT 6-hu GAPDH
hu GAPDH	**(4,35 µg/µl)**
	Plasmid $_P$ DNR-LIB-hu Mip3a(CCL20) Nr. 365
hu CCL20	**5020 bp**
	2360ng/µl

The PCR programm applied for LightCycler for Universal ProbeLibrary UPL probes was as following:

Table 10 PCR-program for LightCycler.

Programm	Function	Temperature	Time	Cycles
Pre-incubation	Activation	95 ºC	10 min	1 cycle
Touchdown	Denaturation	95 ºC	5 sec	10 cycles
	Annealing	65 ºC	5 sec	
	Annealing	62 ºC	10 sec	
Amplification	Denaturation	95 ºC	15 sec	45 cycles
	Annealing	55 ºC	30 sec	
	Amplification	62 ºC	30 sec	

At the end, the RT-PCR product can be quantified depending on the known threshold cycle (Ct) and the known standard curve.

7. Results

In this study we examined the possible response of HPV-transformed (non-malignant as well as malignant cells) and normal human keratinocytes to the cytokines TNFα and IFNβ.

Major interest was to explore differences in expression of the transcription factor IRF7. Eight cell lines including HPV-negative primary keratinocytes, *in vitro* transformed HPV-positive keratinocytes and HPV-positive cervical carcinoma cells were stimulated with TNFα, IFNβ or in combination for 16 hours. RNA was isolated, cDNA was synthesized and quantitative RT-PCR was done to investigate the expression of four genes comprising IRF7, Mx1 as a further IFNβ-responsive gene, CCL20 as a further TNFα-responsive gene and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a house keeping gene. Gene expression of IRF7, Mx1 and CCL20 was normalized to GAPDH. Expression of IRF7, Mx1 and CCL20 in relation to GAPDH of non-stimulated cells was set at 1.

7.1 Evaluation of IRF7 expression in primary human keratinocytes

EXLN and NFK were treated with 1×10^3 U/ml TNFα and/or 1×10^2 U/ml IFNβ for 16 hours. mRNA expression of IRF7 was quantified by quantitative RT-PCR. Stimulation with TNFα only slightly induced IRF7 in both cell types. IFNβ increased IRF7 expression 5-fold in EXLN cells and significantly 8-fold in NFK13 cells (***$p \leq 0.001$) compared to non-stimulated cells. The co-stimulation with TNFα and IFNβ led to the strongest induction of IRF7 in both cell types reaching significantly about 8-folds in EXLN cells and more than 15-fold in NFK13 (***$p \leq 0.001$) (Fig. 10).

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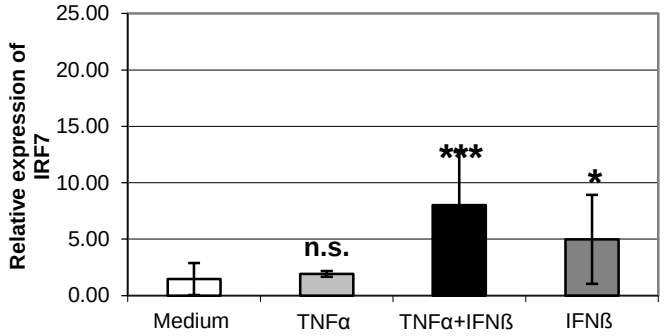

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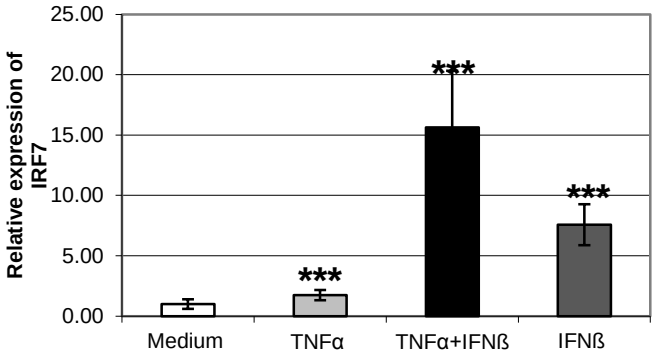


Figure 10 IRF7 expression in HPV-negative primary keratinocytes after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 EXLN (A) or NFK (B) cells were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for IRF7 in relation to GAPDH. Shown are the mean values from three independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (*p ≤ 0.05, ***p ≤ 0.001). n.s. not significant.

7.2 Evaluation of IRF7 expression in *in-vitro* transformed HPV16 or 18 keratinocytes

HPK1A cell line (HPV16-positive) stimulated with TNFα showed very slight induction of IRF7 expression. Up to 14-fold significant increase was observed when cells were treated with IFNβ and 16-fold in response to both stimuli (***p ≤ 0.001) (Fig. 11A).

K51 (HPV18-positive) stimulated with TNFα expressed significantly IRF7 more than 2-fold. IRF7 expression increased significantly to about 10-fold in response to IFNβ and almost 14-fold in response to TNFα and IFNβ together (***p ≤ 0.001) (Fig. 11B).

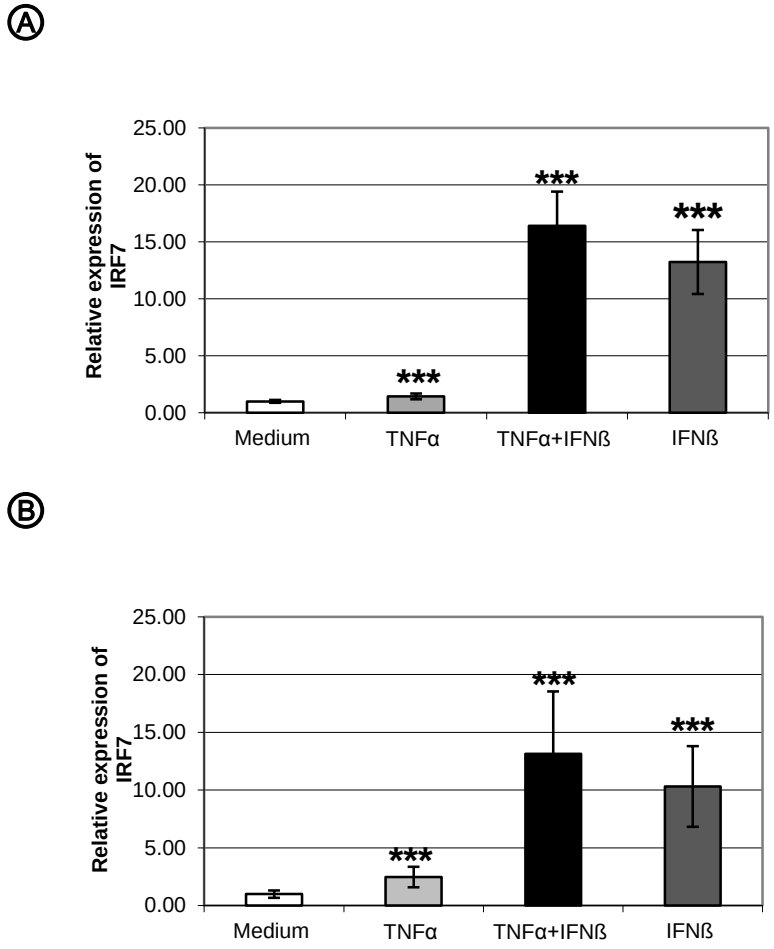
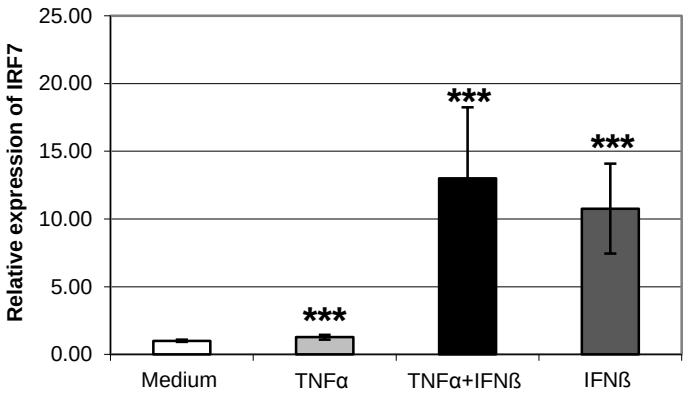


Figure 11 IRF7 expression in *in vitro* immortalized human keratinocytes after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 HPK1A (A) or K51 (B) cells were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for IRF7 in relation to GAPDH. Shown are the mean values from three independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (***p ≤ 0.001).

7.3 Evaluation of IRF7 expression in HPV16-or 18-positive cervical cancer cell lines

Different HPV16- and HPV18-positive cervical cancer cell lines were stimulated with TNFα, IFNβ or in combination and IRF7 expression was analyzed. Treatment of HPV16-positive human cervical carcinoma cells CaSki with TNFα did not trigger a notable induction of IRF7 expression. This expression increased significantly 11-fold after stimulation with IFNβ alone and approximately 14 folds after co-stimulation with TNFα and IFNβ (***p ≤ 0.001) (Fig. 12A). The similar pattern was observed for SW756 cell lines (Fig. 12C). IRF7 expression in HPV18-positive human cervical carcinoma cells C4-I showed significantly an induction up to 4-fold in response to TNFα, 2-fold in response to IFNβ and 5 folds after co-stimulation with both cytokines (***p ≤ 0.001) (Fig. 12B). Furthermore, IRF7 expression was also elucidated in human adenocarcinoma HeLa. TNFα only slightly induced IRF7 expression. This induction reached significantly 8-fold after stimulation with IFNβ (***p ≤ 0.001). When the cells were treated with both stimuli, IRF7 expression raised insignificantly up to 4-fold (Fig. 12D).

Ⓐ



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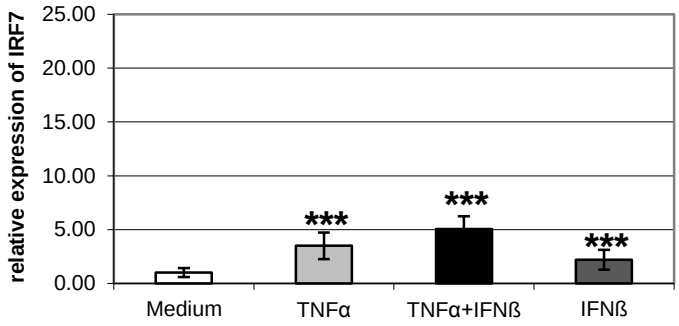
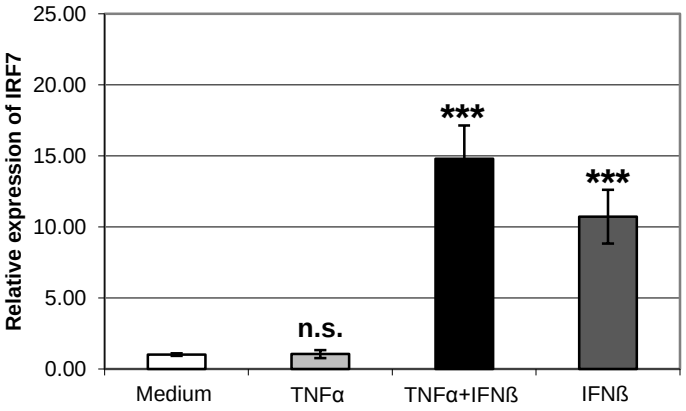

relative expression of IRF7
25.00
20.00
15.00
10.00
5.00
0.00
***
***
***
Medium
TNFα
TNFα+IFNß
IFNß

Ⓒ


Relative expression of IRF7
25.00
20.00
15.00
10.00
5.00
0.00
n.s.
***
***
Medium
TNFα
TNFα+IFNß
IFNß

Ⓓ

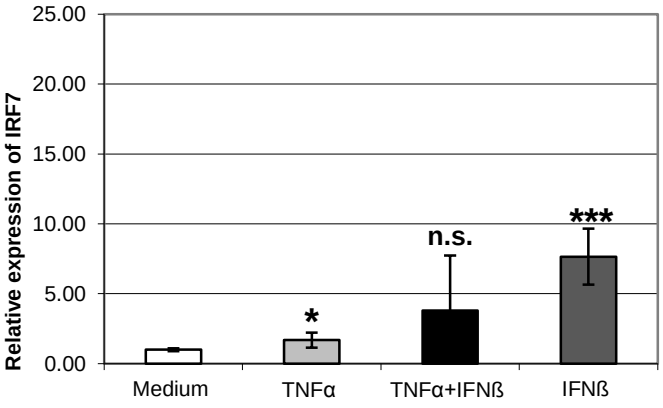

Relative expression of IRF7
25.00
20.00
15.00
10.00
5.00
0.00
*
n.s.
***
Medium
TNFα
TNFα+IFNß
IFNß

Figure 12 IRF7 expression in HPV16-or 18-positive cervical cancer cell lines after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 CaSki (A), C4-I (B), SW756 (C) or HeLa cells (D) were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for IRF7 in relation to GAPDH. Shown are the mean values from three independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (*$p \leq 0.05$; ***$p \leq 0.001$). n.s. not significant.

In summary, the IRF7 response in HPV-positive cells was compared to that obtained in NFK (Fig. 13).

In all TNFα-treated cells, IRF7 induction was at the similar level as for NFK, except C4-I and SW756 cell lines. In C4-I the IRF7 expression was significantly higher, while in SW756 significantly lower.

The similar pattern was observed for TNFα/IFNβ synergism, which was significantly less effective only for C4-I cells, compare to NFK.

IFNβ-mediated increase in IRF7 expression was significantly higher only in HPK1A cells, while in C4-I strongly reduced. For other HPV-positive cell lines, the IFNβ-mediated response was at least at the same level as for NFK.

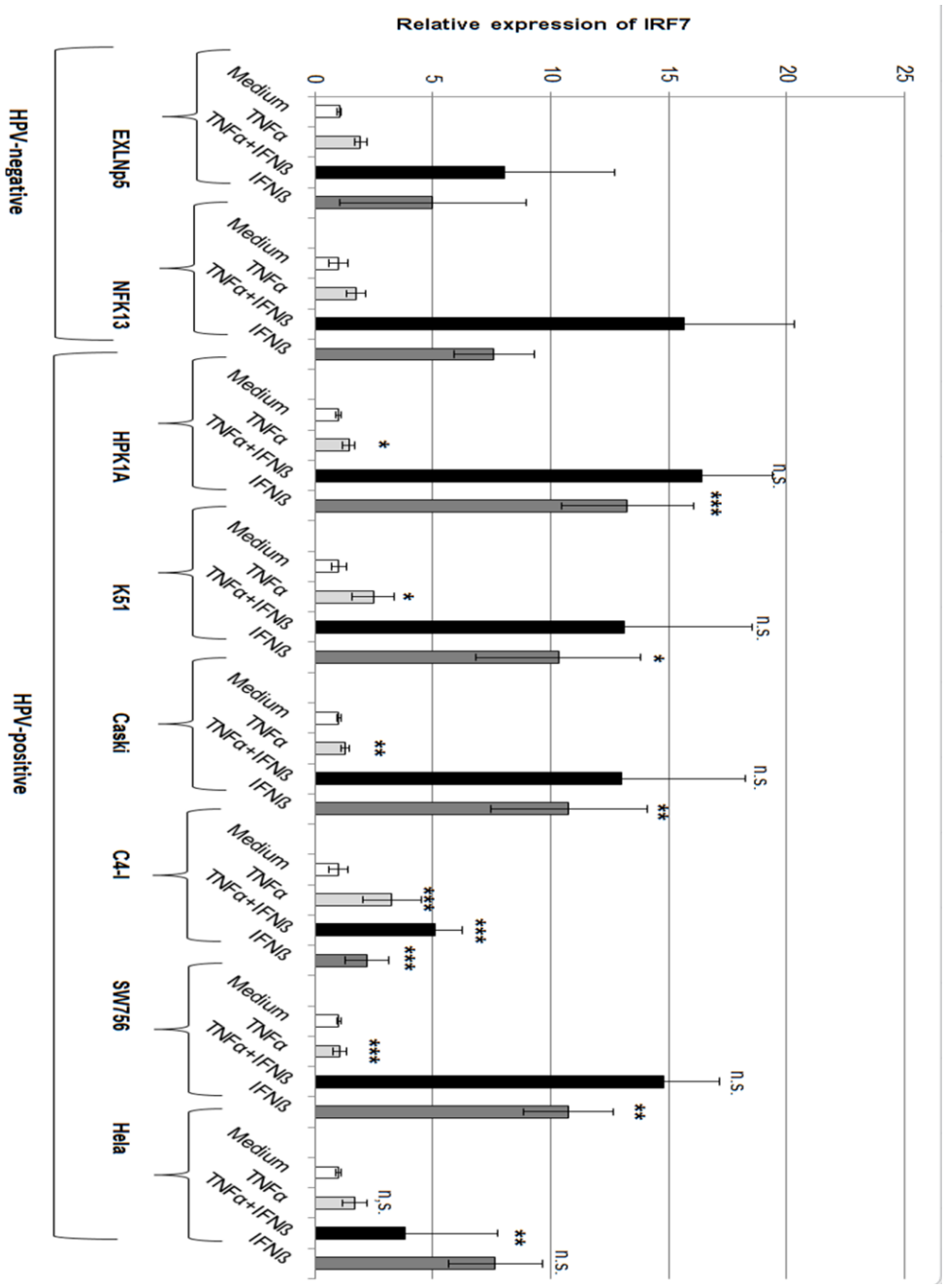
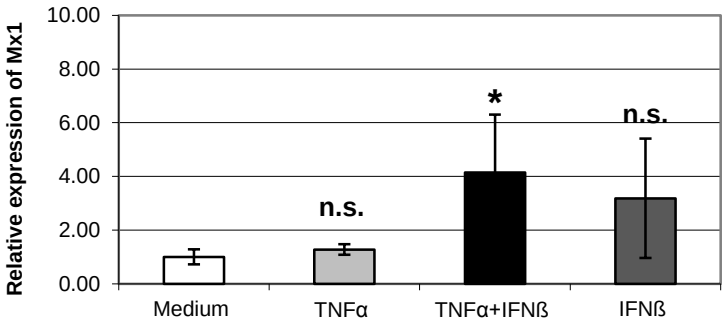


Figure 13 TNFα and IFNβ-mediated IRF7 induction in mucosal human keratinocytes. Asterisks represent statistical significance (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). n.s. not significant.

7.4 Evaluation of Mx1 expression in primary human keratinocytes

EXLN and NFK were treated with 1×10^3 U/ml TNFα and/or 1×10^2 U/ml IFNβ for 16 hours. mRNA expression of Mx1 was quantified by quantitative RT-PCR. No notable induction of Mx1 expression was observed after stimulation with TNFα. When the cells were stimulated with IFNβ, Mx1 expression increased up to 3-fold in EXLN and more than 20-fold in NFK. Stimulation with both stimuli increased Mx1 expression 4-fold in EXLN and significantly 20-fold in NFK (Fig. 14).

Ⓐ



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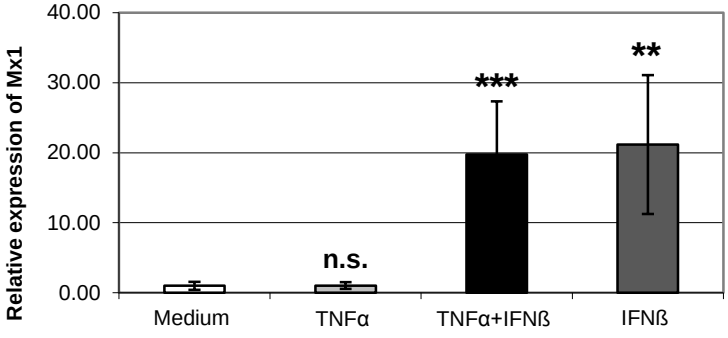


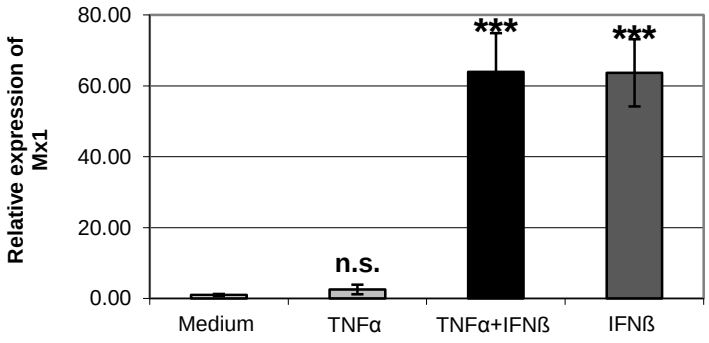
Figure 14 Mx1 expression in HPV-negative primary keratinocytes after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 EXLN (A) or NFK (B) cells were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for Mx1 in relation to GAPDH. Shown are the mean values from three independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). n.s. not significant.

7.5 Evaluation of Mx1 expression in *in-vitro* transformed HPV16 or 18 keratinocytes

HPK1A (HPV16-positive) showed no induction of Mx1 expression upon stimulation with TNFα. Mx1 expression increased significantly 60-fold in response to treatment with IFNβ only or plus TNFα (***p ≤ 0.001) (Fig. 15A).

K51 (HPV18-positive) keratinocytes treated with TNFα expressed Mx1 3-fold. When the cells were stimulated with IFNβ only or plus TNFα, Mx1 expression increased significantly up to 14-fold (***p ≤ 0.001) (Fig. 15B).

Ⓐ



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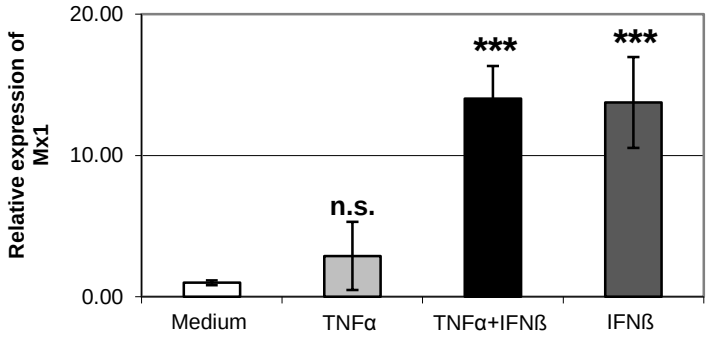


Figure 15 Mx1 expression in *in vitro* immortalized human keratinocytes after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 HPK1A (A) or K51 (B) cells were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for Mx1 in relation to GAPDH. Shown are the mean values from three independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (***p ≤ 0.001). n.s. not significant.

7.6 Evaluation of Mx1 expression in HPV16-or 18-positive cervical cancer cell lines

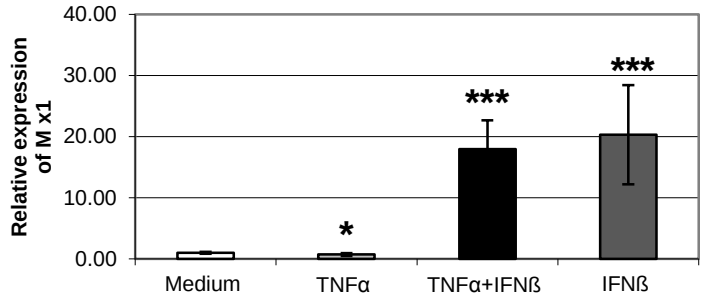
CaSki (HPV16-positive) showed no induction of Mx1 after treatment with TNFα. The cells expressed Mx1 significantly 19-fold in response to IFNβ and 20-fold in response to both stimuli (***p ≤ 0.001) (Fig. 16A).

C4-I (HPV18-positive) did not express an induction of Mx1 after stimulation with TNFα, IFNβ or in combination (Fig. 16B).

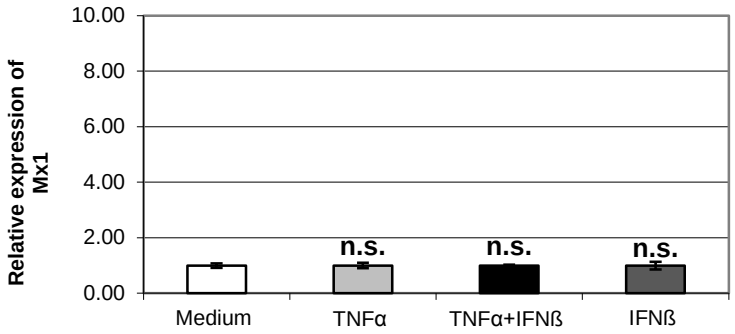
No induction of Mx1 expression was observed in SW756 (HPV18-positive) treated with TNFα. Stimulation with IFNβ only or plus TNFα induced significantly Mx1 expression 70-fold (***p ≤ 0.001) (Fig. 16C).

HeLa (HPV18-positive) stimulated with TNFα expressed Mx1 2-fold. After treatment with IFNβ, Mx1 expression increased 6-fold. A significant induction of Mx1 expression was observed in HeLa cells treated with TNFα and IFNβ (***p ≤ 0.001) (Fig. 16D).

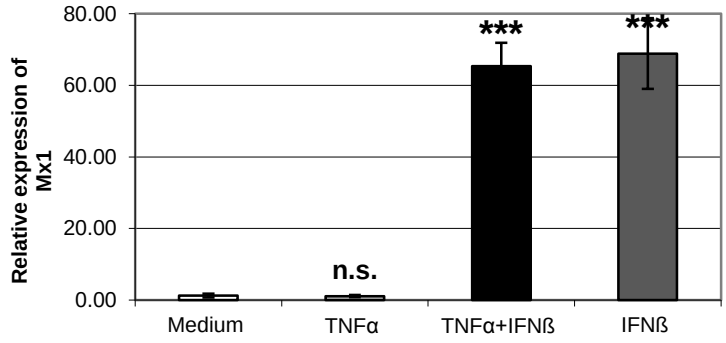
Ⓐ


Relative expression of M x1
40.00
30.00
20.00
10.00
0.00
*
***
***
Medium
TNFα
TNFα+IFNß
IFNß

Ⓑ


Relative expression of Mx1
10.00
8.00
6.00
4.00
2.00
0.00
n.s.
n.s.
n.s.
Medium
TNFα
TNFα+IFNß
IFNß

Ⓒ


Relative expression of Mx1
80.00
60.00
40.00
20.00
0.00
n.s.
***
***
Medium
TNFα
TNFα+IFNß
IFNß

Ⓓ

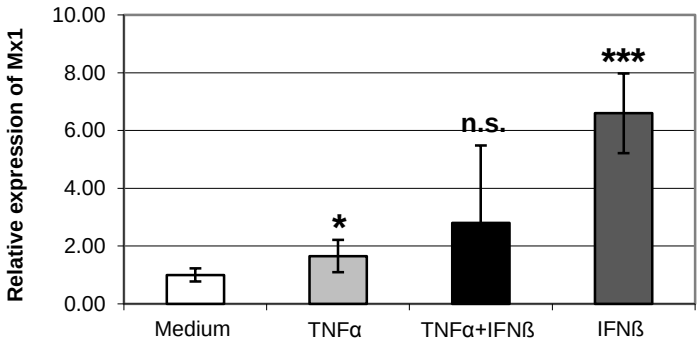


Figure 16 Mx1 expression in HPV16-or 18-positive cervical cancer cell lines after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 CaSki (A), C4-I (B), SW756 (C) or HeLa (D) cells were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for Mx1 in relation to GAPDH. Shown are the mean values from three independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (*$p \leq 0.05$; ***$p \leq 0.001$). n.s. not significant.

In summary, the Mx1 response in HPV-positive cells was compared to that obtained in NFK (Fig. 17).

No notable induction of Mx1 expression was observed in all TNFα-treated cells as for NFK.

Except K51 and HeLa cells, the cells expressed IFNβ-mediated Mx1 at least at the same level as for NFK. IFNβ-mediated increase in Mx1 expression was significantly higher only in SW756 cells.

The similar pattern was observed for TNFα/IFNβ synergism, which was significantly more effective for HPK1A and SW756 cells, compare to NFK.

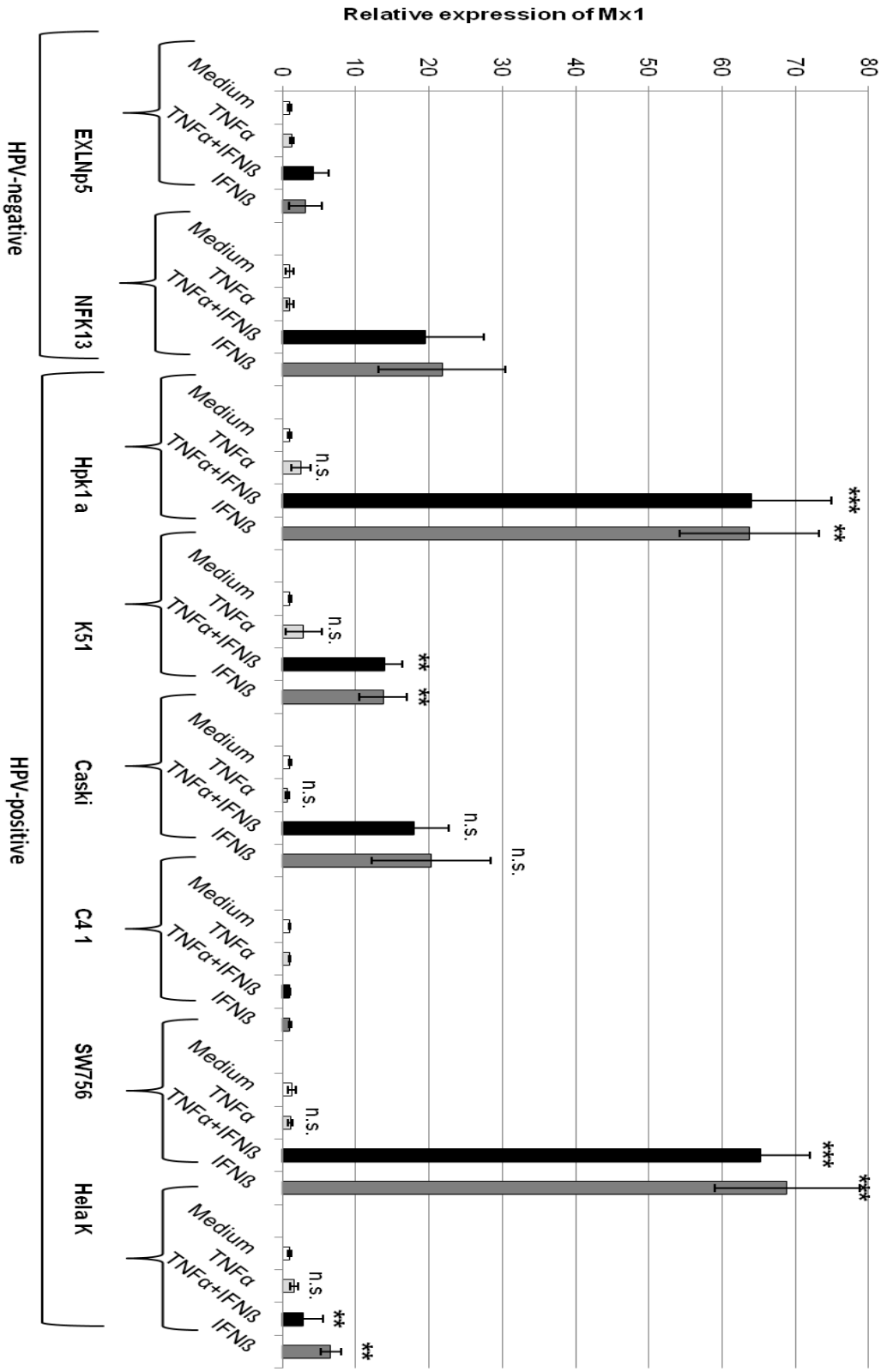

Relative expression of Mx1
0
10
20
30
40
50
60
70
80
HPV-negative
HPV-positive
EXLNp5
NFK13
Hpk1a
K51
Caski
C41
SW756
HelaK
Medium
TNFα
TNFα+IFNß
IFNß
n.s.
**
***

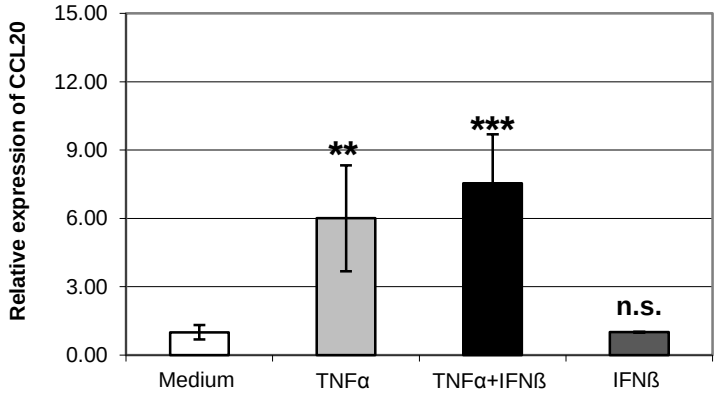
Figure 17 TNFα and IFNβ-mediated Mx1 induction in mucosal human keratinocytes. Asterisks represent statistical significance (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). n.s. not significant.

7.7 Evaluation of CCL20 expression in primary human keratinocytes

An induction 6-fold of CCL20 expression in EXLN keratinocytes was observed in response to TNFα. The induction increased significantly 8-fold in cells treated with TNFα and IFNβ (***p ≤ 0.001). No response was seen upon stimulation with IFNβ (Fig. 18A)**.**

Stimulation of NFK keratinocytes with TNFα only or plus IFNβ caused significantly an induction of CCL20 expression about 5 folds (***p ≤ 0.001). A suppression of CCL20 expression occurred when the cells were treated with IFNβ only (Fig.18B).

Ⓐ



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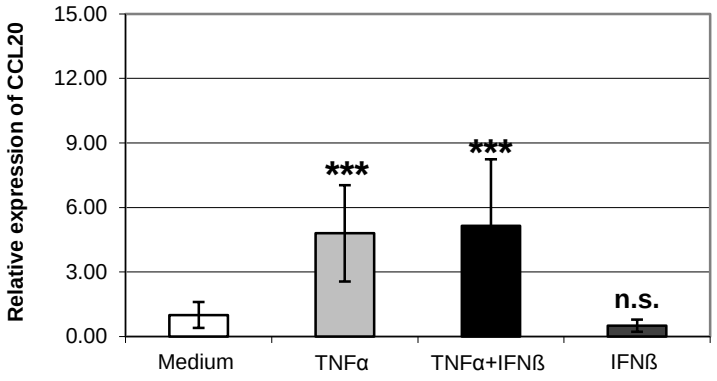


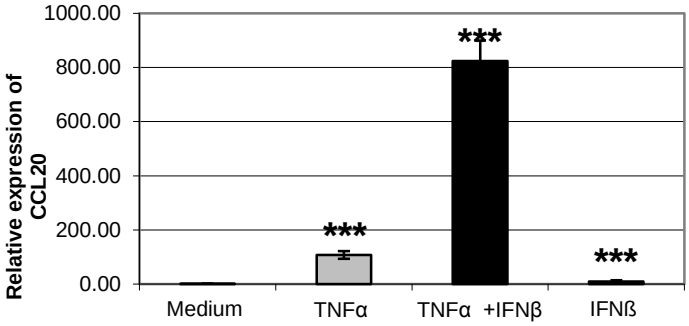
Figure 18 CCL20 expression in HPV-negative primary keratinocytes after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 EXLN (A) or NFK (B) cells were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for CCL20 in relation to GAPDH. Shown are the mean values from three independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (**p ≤ 0.01; ***p ≤ 0.001). n.s. not significant.

7.8 Evaluation of CCL20 expression in *in-vitro* transformed HPV16 or 18 keratinocytes

HPK1A keratinocytes expressed CCL20 significantly 110-fold in response to TNFα, 820-fold in response to TNFα accompanied with IFNβ and up to 10-fold after treatment with IFNβ only (***p ≤ 0.001) (Fig. 19A).

K51 keratinocytes showed an induction of CCL20 expression 10-fold after stimulation with TNFα and 8-fold upon stimulation with TNFα plus IFNβ. The cells treated with IFNβ showed no induction of CCL20 expression (Fig.19B).

Ⓐ



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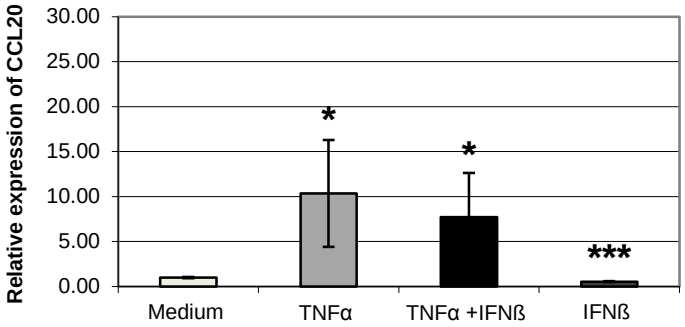


Figure 19 CCL20 expression in *in vitro* immortalized human keratinocytes after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 HPK1A (A) or K51 (B) cells were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for CCL20 in relation to GAPDH. Shown are the mean values from two independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (*p ≤ 0.05; ***p ≤ 0.001).

7.9 Evaluation of CCL20 expression in HPV16-or 18-positive cervical cancer cell lines

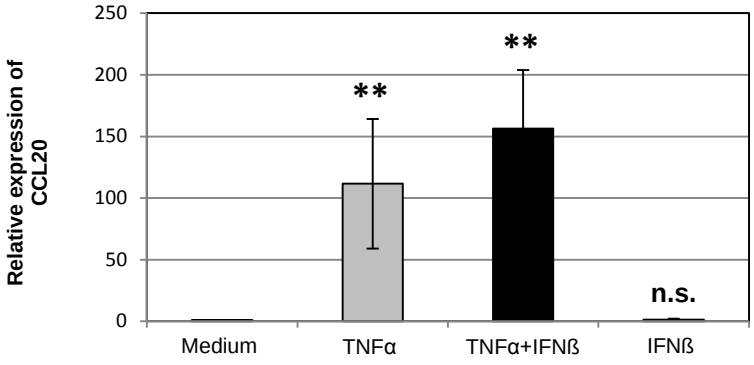
CaSki keratinocytes stimulated with TNFα expressed CCL20 110-fold. An induction of CCL20 expression up to 155-fold was observed after co-stimulation with TNFα and IFNβ. Treatment with IFNβ only caused no induction of CCL20 expression (Fig. 20A).

In response to TNFα, CCL20 expression increased 20-fold in C4-I cells. CCL20 expression raised 30-fold, when the cells were treated with TNFα and IFNβ. After stimulation with IFNβ, CCL20 expression was induced 6-fold (Fig. 20B).

SW756 keratinocytes showed an induction of CCL20 expression 39-fold in response to TNFα. Co-stimulation with TNFα and IFNβ increased CCL20 125-fold. CCL20 expression raised 2-fold in cells treated with IFNβ (Fig. 20C).

HeLa keratinocytes expressed CCL20 37-fold in response to TNFα and significantly up to 40-fold after treatment with TNFα and IFNβ. No induction was observed in HeLa cells stimulated with IFNβ (Fig. 20D).

Ⓐ



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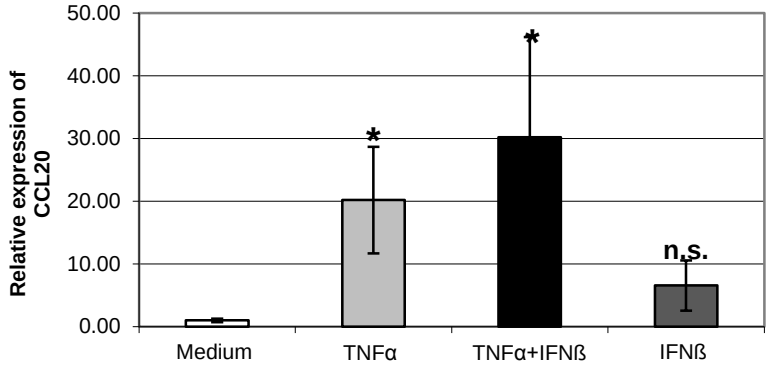

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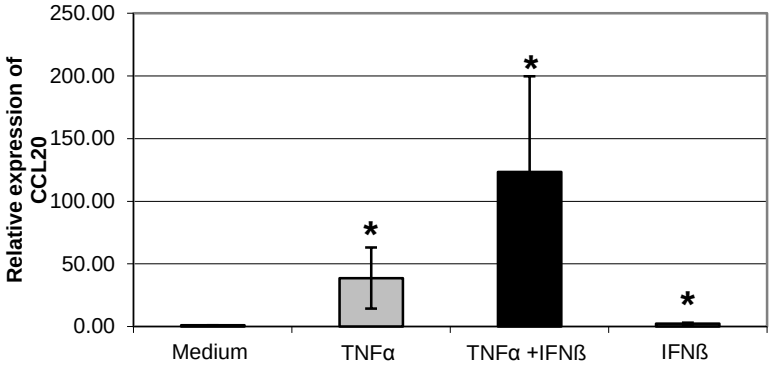

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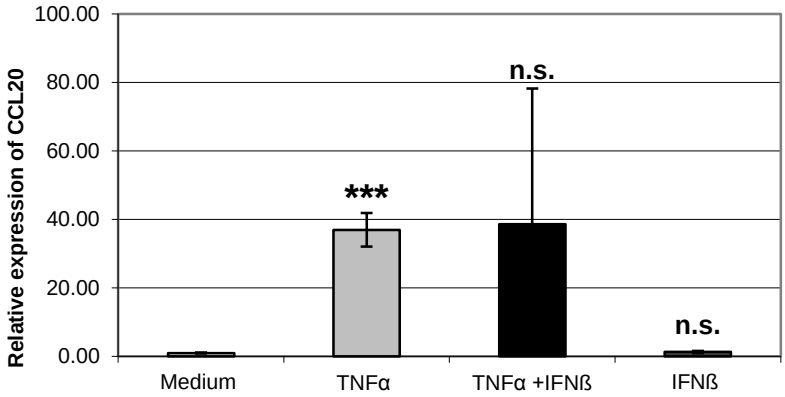

Figure 20 CCL20 expression in HPV16-or 18-positive cervical cancer cell lines after stimulation with TNFα, IFNβ or co-stimulation. 1×10^6 CaSki (A), C4-I (B), SW756 (C) or HeLa (D) cells were stimulated with medium, TNFα (1×10^3 U/ml), IFNβ (1×10^2 U/ml) and TNFα plus IFNβ. After 16 hours mRNA was isolated from the cells and quantified by real-time PCR for CCL20 in relation to GAPDH. Shown are the mean values from two independent experiments performed in duplicates ± SD. Asterisks represent statistical significance (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). n.s.not significant.

In summary, the CCL20 response in HPV-positive cells was compared to that obtained in NFK (Fig. 21).

All cells treated with TNFα expressed CCL20, significantly only in HPK1A and HeLa cells, more than NFK.

Except K51, the similar pattern was observed for TNFα/IFNβ synergism, which was significantly more effective for HPK1A, compare to NFK.

HPK1A cells treated with IFNβ expressed significantly CCL20 more than NFK, while other cells did not respond to the treatment with IFNβ.

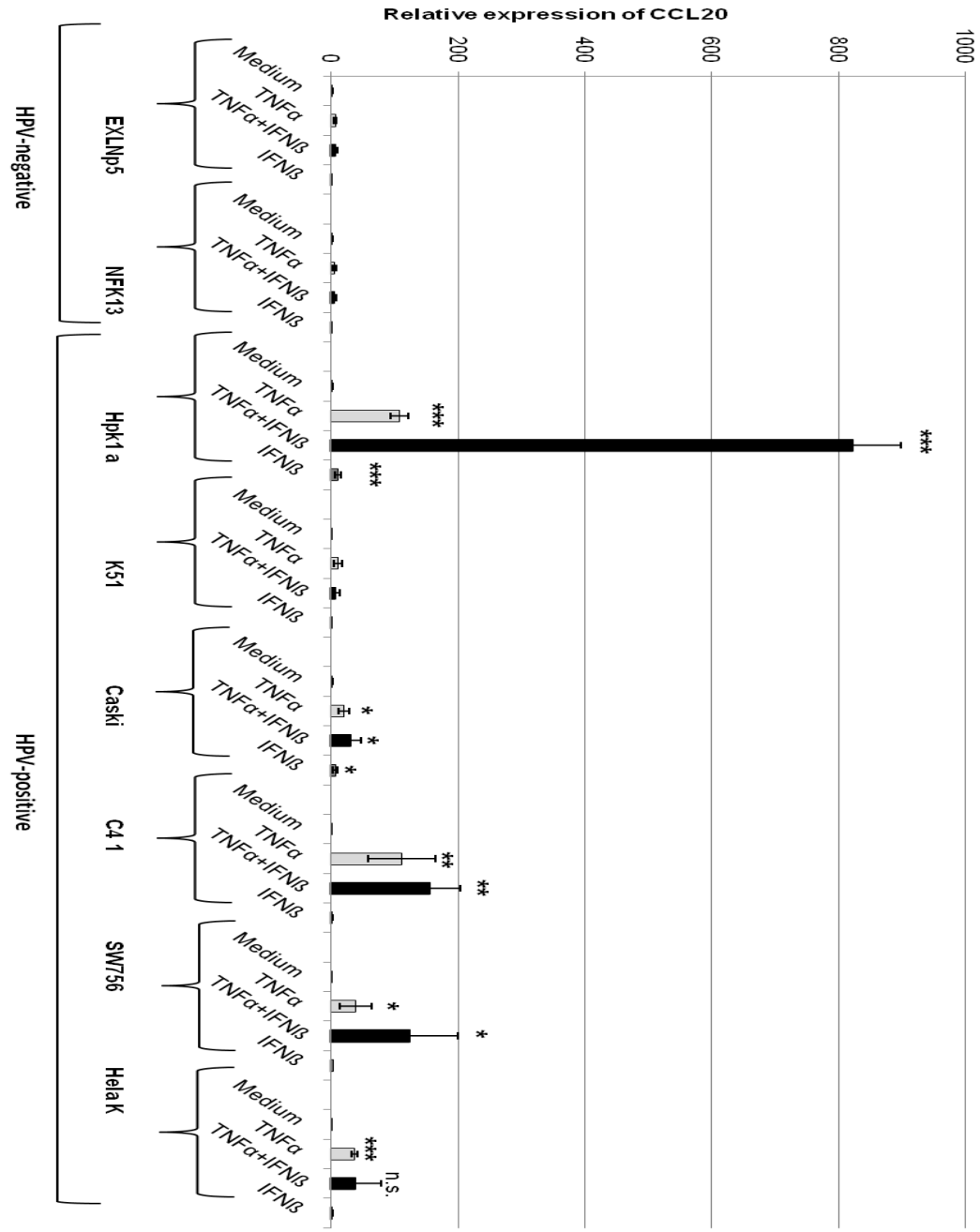

Figure 21 TNFα and IFNβ-mediated CCL20 induction in mucosal human keratinocytes. Asterisks represent statistical significance (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). n.s. not significant.

7.10 Comparison between IRF7 expression and Mx1 expression in HPV negative and HPV-positive keratinocytes

Our results corresponded to the previous observations which referred firstly to the ability of interferon beta to induce Mx1 expression and secondly to the inability of TNFα to do that (Holzinger et al, 2007; Aebi et al, 1989; Horisberger, 1992; Simon et al, 1991). Compared to Mx1 expression, it could be noticed that the IRF7 expression was slightly induced after stimulation with TNFα and the highest induction was reported in C4-I cells. In the context of investigating of the effects of interferon beta on the IRF7 expression in comparison with an IFN-inducible gene (Mx1), our findings referred to an induction of IRF7 expression less than Mx1 expression except in three cell lines (EXLN, C4-I, and HeLa). The similar pattern was observed after co-stimulation with TNFα and IFNβ (Fig. 22).

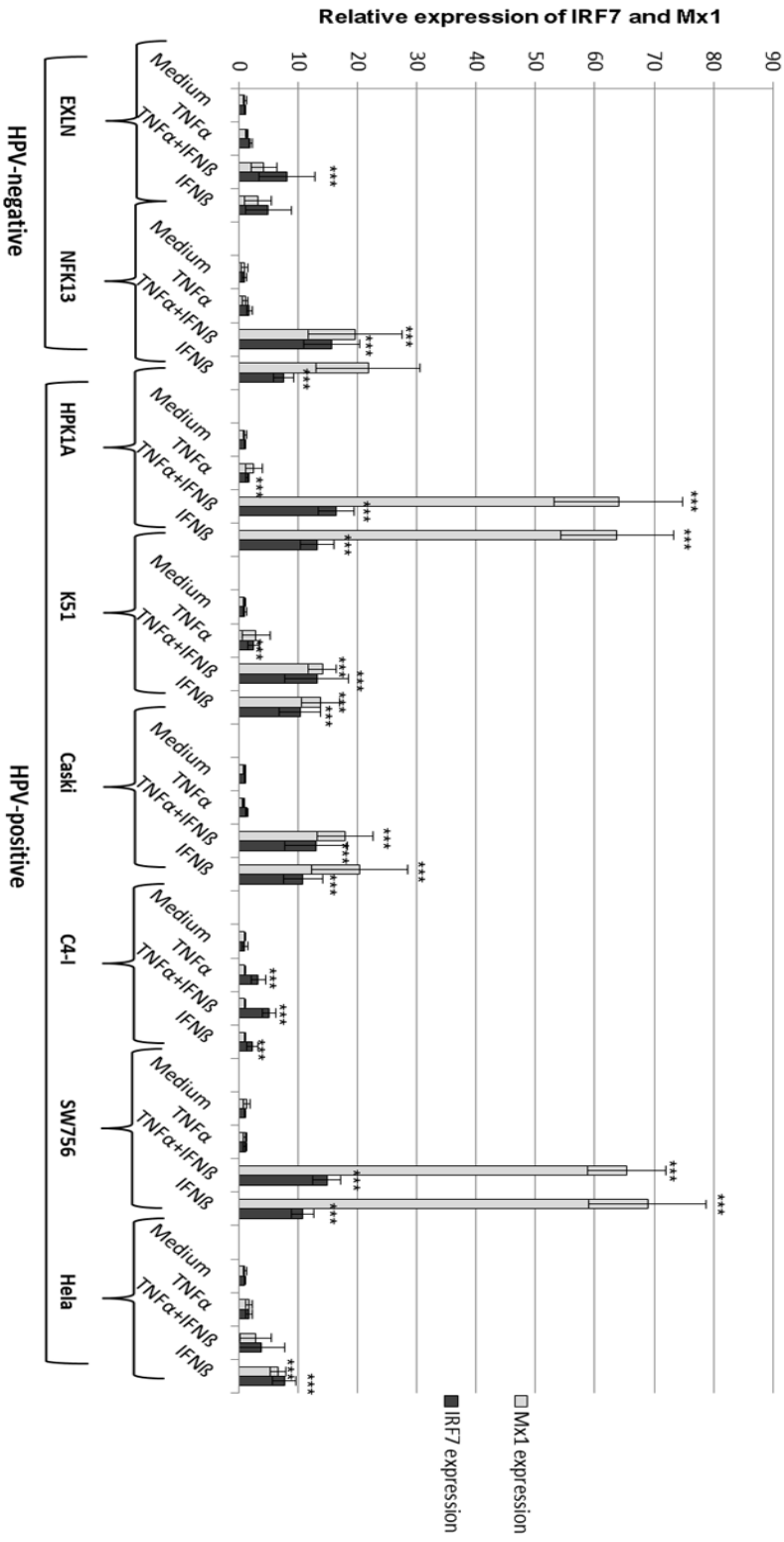
Relative expression of IRF7 and Mx1
0
10
20
30
40
50
60
70
80
90
Medium
TNFα
TNFα+IFNß
IFNß
EXLN
NFK13
HPK1A
K51
Caski
C4-I
SW756
Hela
HPV-negative
HPV-positive
Mx1 expression
IRF7 expression

Figure 22 TNFα and IFNβ-mediated IRF7 induction compared to TNFα and IFNβ-mediated Mx1 in mucosal human keratinocytes. Asterisks represent statistical significance (***p ≤ 0.001).

7.11 Comparison between IRF7 expression and CCL20 expression in HPV-negative and HPV-positive keratinocytes

According to the comparison between IRF7 expression and CCL20 expression in all studied cell lines, we could report that the stimulation with TNFα caused an increasing in IRF7 expression less than CCL20 expression in all cells. In contrast, IFNβ-mediated IRF7 expression was greater than IFNβ-mediated CCL20 expression in all cell lines. Moreover, the treatment with both stimuli TNFα and IFNβ increased IRF7 expression more than CCL20 expression in all cell lines except in EXLN, NFK and K51 cells (Fig. 23).

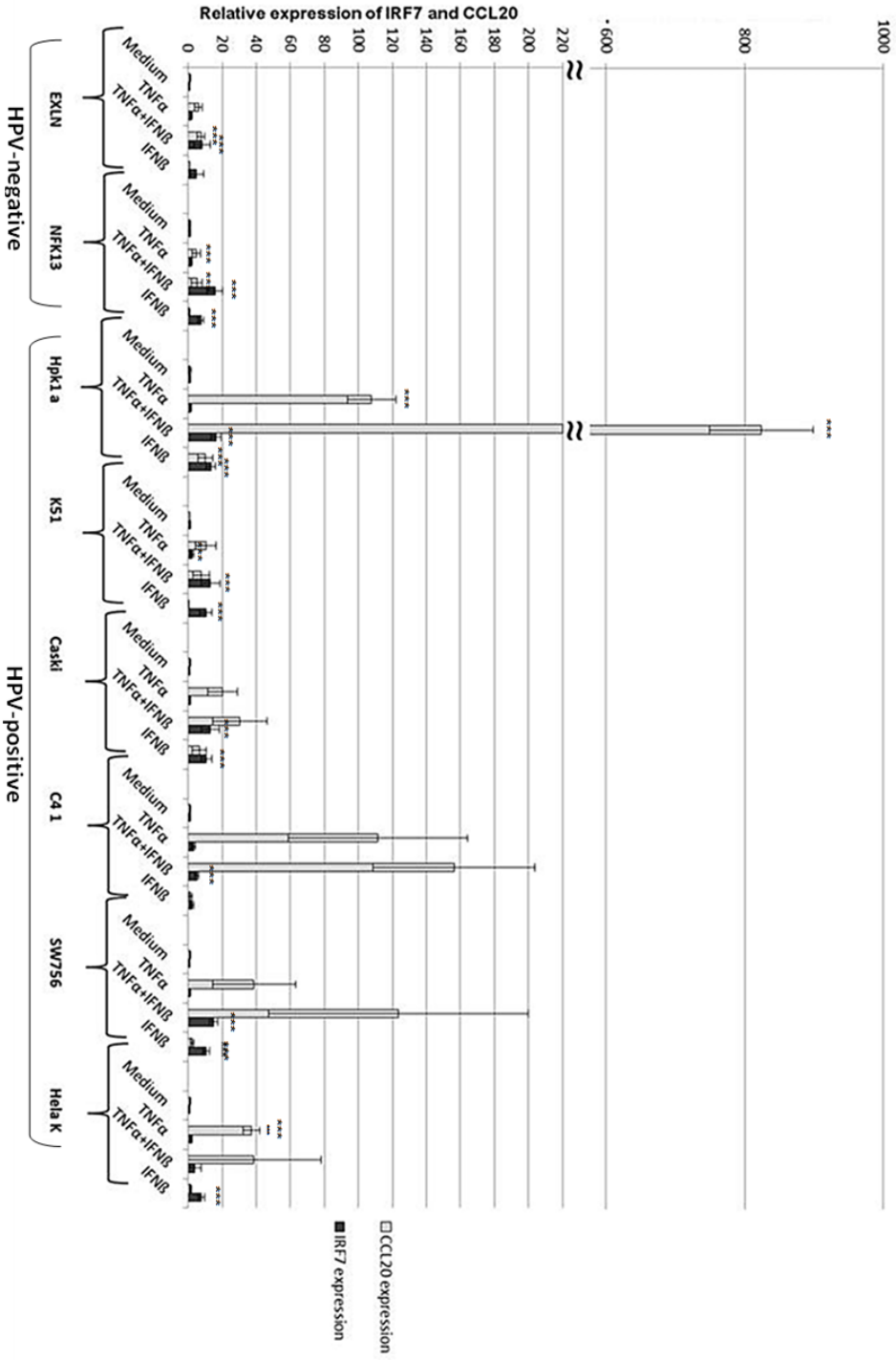

Figure 23 TNFα and IFNβ-mediated IRF7 induction compared to TNFα and IFNβ-mediated CCL20 in mucosal human keratinocytes. Asterisks represent statistical significance (***p ≤ 0.001).

8. Discussion

More than 99% of cervical carcinomas harbour HPV, especially HPV16 and HPV18 and these both genotypes are together responsible for 70% of cervical carcinomas (Bosch et al, 2008; Zur Hausen, 1990). Accordingly, extensive studies have been carried out to investigate how this virus can escape the immune system and persist in the cervical epithelium eventually leading to cancer. Cytokines, particularly IFNs, are an important part of the host defense against viral infection. It has been proposed that oncoproteins of high-risk HPV can suppress type I IFN signaling.

Extensive attempts have been undertaken to study the impact of treatment of HPV-induced lesions with exogenous IFNs. Different observations were made. Lesions comprising episomal DNA have been completely removed, while lesions with integrated copies of viral DNA have been resistant (Beglin et al, 2009; Koromilas et al, 2001).

HPV is double-stranded DNA virus. It has been speculated that its genome can be recognized by TLR9 leading to activate the MyD88-dependent pathway.

In vitro, there are indications that HPV-induced triggering of TLR9 responses occurs (Hasan et al, 2007). TLRs activation, in turn, has been shown to induce IRF7 (Fig. 5) (Kawai & Akira, 2010). Upon viral infection IRF3 may be phosphorylated as well translocated resulting in production of IFNα and IFNβ which induce the IRF7 expression (Yoniyama et al, 1998; Juang et al, 1998; Sato et al, 2000; Lin et al; 1998; Sato et al, 1998).

Numerous previous studies have reported that IFNβ can induce the IRF7 expression through the JAK-STAT signaling pathway by forming ISGF3 complex (STAT1, STAT2, and IRF9) on ISRE (Fig. 3) (Silvennoinen et al, 1993; Katze et al; 2002; Velazquez et al, 1992; Darnell et al, 1994; Koromilas et al, 2001). Research has extensively concentrated on the mechanisms by which HPV can modify the cellular response to the treatment with cytokines such as TNFα and IFNβ. Of note, our group has recently shown that IRF7 binds to the HPV DNA

non-coding region thereby inducing HPV8 late promoter (Oldak et al, 2011). Thus, IRF7 is not only part of an antiviral response but may also have positive effects on HPV gene expression.

Lu and others (2002) have reported previously that TNFα can induce IRF7 expression in human peripheral blood monocytes through NF-κB signaling pathway.

This study showed that TNFα has only limited ability to induce IRF7 expression in normal primary keratinocytes, *in vitro* HPV16- or HPV18-immortalized keratinocytes, HPV18-positive human adenocarcinoma HeLa and in HPV18-positive human cervical carcinoma C4-I cells. No induction was found in HPV18-positive cells of a differentiated cervical squamous cell carcinoma (SW756) and HPV16-positive human cervical carcinoma cells (CaSki). This may indicate that HPV16 has the ability to disrupt the TNFα signaling pathway. In productively infected epithelium HPV DNA exists as episomal genome. Here, the function of the E2 protein is retained repressing both oncoproteins E6 and E7. In contrast, in malignant cells, most often integration of viral DNA into host genome takes place in the region of the E2 gene resulting in disruption of its function. This leads to the upregulation of E6 and E7 oncoproteins which promote malignant transformation (Tonon et al, 2001; Dowhanick et al, 1995). Moreover, it has been observed that the HPV16 E6 and E7 reduce the activity of NF-κB in HPV16-positive human cervical carcinoma cells (CaSki) (Havard et al, 2002)**.** In agreement with the past findings, our data showed suppression of the IRF7 expression in HPV16-positive human cervical carcinoma cells (CaSki). On the other hand, we observed suppression of IRF7 expression in HPV18-positive human adenocarcinoma HeLa in comparison with the HPV-negative keratinocytes and HPV18-positive keratinocytes. Also, we observed an abrogation of the IRF7 expression in HPV18-positive cells of differentiated cervical squamous cell carcinoma (SW756). One can assume that the high levels of HPV18 or 16 E6 and E7 in the cervical cancer cells interfere with the TNFα signaling pathway. Only in C4-I cervical cancer cells and HPV-18 transformed

K51 cells, IRF7 expression was significantly stronger than in HPV-negative keratinocytes and HPV18-positive keratinocytes.

Similarly, the effect of IFNβ on the IRF7 expression in the presence or absence of HPV was tested. Our data showed that the IFNβ had the capability to activate the IRF7 expression in HPV-negative primary keratinocytes, *in vitro* HPV16- or HPV18-immortalized keratinocytes and in HPV16- or HPV18-positive cancerous keratinocytes. However, the levels of the induction were different. In all tested HPV-positive carcinoma cell lines, IFNβ-mediated IRF7 induction was at least as good as in NFK. Exceptions were C4-I cells. In HeLa cells and C4-1 cells the TNFα/IFNβ synergism was also less effective. We demonstrated that in HPV16- or HPV18-positive cells the IFNβ-mediated induction of IRF7 expression was in some cases even higher than in the HPV-negative cells. This was obviously not due to episomal HPV genomes, since both in *in vitro* transformed as well as HPV-positive cancerous keratinocytes the viral DNA is integrated into the host genome. In most cases this integration is associated with deletion of regions including the E1, E2, E4, and E5 open reading frames (ORFs) and retention of E6 and E7 genes (Shirasawa et al, 1987). In this context, an interest study has shed light on the role of HPV16 E5 in IFNβ signaling pathway and it has reported that HPV16 E5 induces IFNβ expression through stimulation of IRF1 (Muto et al, 2011). Consistent with the mentioned study, our data showed high expression of IRF7 in HPV16 positive keratinocytes HPK1A. This may be because of the expression of E5 which has been recently identified as a positive regulator of the IFNβ expression via induction of IRF1. This hypothesis should, however, be tested. Moreover, it can be assumed that this did not account for the slightly stronger inducibility in the cancer cell lines CaSki and SW756.

Barnard and McMillan (1999) have demonstrated the suppression of IFNα-inducible gene expression by HPV16 E7 through the interaction between E7 and IRF9 (p48) which is an important part of the ISGF3, so that this interaction inactivates the ISGR3 complex formation resulting in the hindering of the IFNα signaling pathway. Furthermore, it has been reported that the HPV16 E7 has the

opportunity to bind to IRF1, which is a transcription factor mediates the IFN signaling induced by viral infection or IFN treatment by binding to ISRE in the promoter of IFN-inducible genes causing transcriptional activation. So that, the interference of HPV16 E7 with IRF1 creates repression of the transactivation activity of IRF1 leading to suppression of the response to IFN-treatment (Park et al, 2000; Taniguchi et al, 1998). Moreover, it has been observed that HPV18 E6 binds to Tyk2 blocking the activation of the Jak-Stat pathway by IFNα (Li et al, 1999). In contrast to this, our data demonstrated that in most HPV-positive keratinocytes and cancer cells, IFNβ signaling leading to IRF7 induction is not disturbed.

In addition, we quantified the IRF7 expression by RT-PCR after co-stimulation with TNFα and IFNβ in HPV-negative normal keratinocytes, *in vitro* HPV16- or HPV18-immortalized keratinocytes and in HPV16- or HPV18-positive cancerous keratinocytes. And as a result, we documented that all studied cell lines, except HeLa, presented an IRF7 expression in response to the stimulation with both cytokines TNFα and IFNβ greater than in response to each cytokine separately.

Mx1 is an interferon-stimulated gene which can be induced in response to type I IFN and slightly in response to IFNγ and type III IFN (Holzinger et al, 2007; Aebi et al, 1989; Horisberger, 1992). Contrarily, it has been demonstrated that Mx1 gene dose not respond to the stimulation with TNFα (Simon et al, 1991). In our experiments, we used Mx1 as a positive control to the activity of IFNβ, and we found an induction of this gene expression after treatment with IFNβ alone or in addition to TNFα in all studies cell lines except C4-I cells which did not show any response. This indicated that the response to exogenous IFNβ was retained in most HPV-positive cells not only with respect to IRF7 but also the classical IFN-inducible gene Mx1. Additionally, there was remarkable the big difference in Mx1 expression between the exocervical keratinocytes and foreskin keratinocytes, and this difference may be dependent on the cell phenotype. Our experiments showing Mx1 expression after stimulation with IFNβ alone or combined to TNFα indicated that TNFα has not the ability to reinforce the effect of IFNβ.

In the same way, the expression of CCL20 was investigated in this study as a control of the TNFα bioactivities. It has been previously reported that the expression of CCL20 can be potently induced upon treatment with TNFα through the NF-kB signaling pathway (Reibman et al, 2002; Nakayama et al, 2001; Fujiie et al, 2001; Hosokawa et al, 2005; Tohyama et al, 2001). Consistent with the past findings, our result documented an induction of the CCL20 expression in response to TNFα in all cell lines, however, again with strong differences level of induction particularly was observed in *in vitro* HPV16-immortalized foreskin keratinocytes and also in HPV18-positive cancerous cells C4-I.

In summary, this study suggests that TNFα has limited ability to induce IRF7 expression in HPV-transformed and normal human keratinocytes. This induction is in some cases of HPV18-transformed keratinocytes stronger than in HPV-negative keratinocytes. In addition, our data show that IRF7 expression induced by exogenous IFNβ is retained in most HPV-positive malignant and non-malignant keratinocytes. Except HeLa cells, TNFα and IFNβ induce synergistically IRF7 expression in HPV-negative and HPV-transformed keratinocytes.

The present work demonstrates that the exogenous IFNβ signaling leading to induction of Mx1 expression and TNFα signaling leading to induction of CCL20 are not disturbed in HPV-transformed and normal human keratinocytes.

Thus, HPV16 and HPV18 may have no negative effects on the response of mucosal human keratinocytes to the treatment with TNFα and/or IFNβ.

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